Published in final edited form as:
Int Rev Neurobiol. 2010 ; 91: 25–51. doi:10.1016/S0074-7742(10)91002-7.

The Genetics of Behavioral Alcohol Responses in Drosophila

Aylin R. Rodan[1] and **Adrian Rothenfluh**[2]
Department of Psychiatry and Program in Neuroscience, UT Southwestern Medical Center, Dallas, TX

I. INTRODUCTION

I. A. Alcohol and Alcoholism

Alcohol is one of the most widely abused drugs in the world, and alcohol use disorders have grave consequences for both the affected individual and society at large. Our understanding of what changes in the brain that cause a person to go from casual consumption to uncontrolled use is still lacking. One of the reasons for this is the fact that unlike most other drugs of abuse, ethanol does not have a primary high affinity target in the brain, but rather, alcohol binds to many proteins at relatively low affinities and thus many neurochemical systems may be affected by ethanol and contribute to alcoholism.

Many studies have indicated that alcoholism has a large genetic component (reviewed by Hill, this issue). This makes genetic model organisms like the mouse (Buck, this issue) or Drosophila very relevant as tools to isolate candidate genes altering behavioral alcohol responses and drinking patters, and to understand how these genes work and how they might act to alter brain function in addicted individuals. This review focuses on the advances in our understanding of the genes and biochemical pathways that mediate the behavioral responses to alcohol in flies.

I. B. Drosophila as a Model Organism

Drosophila melanogaster vinegar flies have been used for over a hundred years as a model organism to study the laws and mechanisms of heredity. In this review, the terms Drosophila, or flies, will refer to this species, unless specifically noted otherwise. The main reasons why Thomas Hunt Morgan's research group decided to focus on Drosophila a hundred years ago were threefold: first, flies are easily and inexpensively cultured in glass bottles with banana pulp. Second, their life cycle is fast, requiring only about 12 days at room temperature to go from freshly laid egg to reproducing adult. And third, a single female can have over a hundred offspring. In addition to that, Drosophila larvae have giant salivary gland chromosomes, allowing the visualization of subsegments of the four chromosomes. This was the basis for the accumulation and characterization of many fly strains with cytologically defined chromosomal aberrations, like deficiencies and duplications. These were, and still are, invaluable tools for genetic mapping and stock maintenance techniques. All these reasons have made Drosophila an excellent model organism for the study of the basics of genetic inheritance.

Many fundamental discoveries were made in flies, including genetic recombination, or the fact that X-rays are mutagenic (Sturtevant, 1967). The genomic sequence of *Drosophila melanogaster* was completed in 2000 (Adams et al., 2000), and currently, 192 wild-derived recombinant inbred lines are being fully sequenced, some of which have already been used

[2]corresponding author: Adrian.Rothenfluh@UTSouthwestern.edu.
[1]Current address: Division of Nephrology, UT Southwestern Medical Center, Dallas, TX

to associate genetic variation with behavioral alcohol responses (Morozova et al., 2009) (see section III.B.) Current estimates are that ~75% of genes associated with a human disease have an obvious ortholog in flies (Chien et al., 2002). This high degree of conservation became obvious in the 1980s when many developmental genes found in Drosophila were shown to have similar function in humans (Gehring et al., 2009). However, even in such complex behaviors as circadian rhythm there is a very high degree of conservation between flies and mammals, both in gene structure and molecular function (Collins and Blau, 2007).

While forward genetics, i.e. going from phenotype to gene, has traditionally been the approach taken in Drosophila, genetic transformation was established in 1982 (Rubin and Spradling, 1982). This allows both the reintroduction of a gene to confirm that its mutation causes a phenotype, and also allows for introduction of transgenes mis-, or overexpressing specific proteins or their altered derivatives. One of the most widely used tools in that regard is the binary Gal4/UAS system (Brand and Perrimon, 1993). One transgene carries the yeast transcriptional activator Gal4 under the control of a specific promoter, allowing for spatially controlled expression. The second transgene carries a cDNA of interest under the control of the Gal4-responsive upstream activating sequence (UAS). This allows testing of hypotheses such as: is CNS-specific expression of a cDNA sufficient to rescue the phenotype caused by a given mutation? In addition, certain proteins can be expressed in neurons that induce temperature-sensitive neuronal silencing (Kitamoto, 2001), or action potentials (Pulver et al., 2009), allowing questions like: is a given set of neurons necessary, or even sufficient for a certain behavioral response? Given that a substantial effort is being made to produce 5000 unique Gal4 drivers expressing in a small subset of CNS neurons (Pfeiffer et al., 2008), we might well learn much more about the neurons and circuits mediating these behaviors in the near future.

In addition to the binary Gal4/UAS system, other reverse genetic tools have been developed in flies that allow going from gene to phenotype. One of them is the systematic generation of UAS-lines expressing interfering RNA constructs for knock-down of every Drosophila gene (Dietzl et al., 2007). And in the last few years, it has also become easier, and more common to use homologous recombination to generate targeted gene knock-outs, or replacement knock-ins of any given fly gene (Maggert et al., 2008). Lastly, large-scale efforts have been undertaken to generate a library of fly strains that contain mutations in every single gene (Matthews et al., 2005).

II. Ethanol Exposure in Flies

I. A. Ethanol Toxicity Studies

Many early studies, reviewed in (Guarnieri and Heberlein, 2003), have focused on the effects of ethanol on larval and adult fly survival, and increases in survival were often labeled tolerance. In this review, the term tolerance is exclusively applied to a decreased ethanol response caused by prior ethanol exposure. Many of these studies focused on the frequency of the ethanol catabolizing enzyme alcohol dehydrogenase, ADH, and two naturally occurring alleles Adh^F, a more active, and Adh^S, a less active variant. While ADH-deficient larvae are very sensitive to ethanol toxicity (David et al., 1976), very little difference was observed between these flies and wild-type controls in ethanol-induced sedation in the inebriometer (Singh and Heberlein, 2000). Furthermore, the more active allele Adh^F did not increase in frequency during a 12 generation selection for sedation-resistance in the inebriometer (Cohan and Graf, 1985), nor was *Adh* differentially expressed in a different set of selectively bred resistant, versus sensitive strains (Morozova et al., 2007). Thus *Adh* plays a significant role in ethanol-toxicity in flies, but has little effect on ethanol-induced sedation.

II. B. Exposing Flies to Alcohol

Flies can be exposed to ethanol in a number of different ways. Mixing alcohol into the food has been the traditional way of exposure to study ethanol resistance and toxicity. Up until recently, feeding had the distinct disadvantage that the amounts of alcohol consumed by the flies was an unknown quantity. With the introduction of the CAFE assay this has changed (Ja et al., 2007). CAFE stands for capillary feeder, and in this paradigm the only available food to the flies is contained within calibrated microcapillaries that allow the experimenter to precisely measure the amounts of ethanol-containing food that has been consumed by the flies. This assay has been used to great success to show that Drosophila like drinking alcohol (Devineni and Heberlein, 2009) (see section II.F.)

Subcutaneous injection of ethanol is routine in rodents, yet even mice are still enormous when compared to Drosophila, a small species even for flies in general. Given that an adult Drosophila has a volume of about 2 μl, it is not surprising that very few studies have used intraabdominal injection as a means of delivery. Nevertheless, direct injection of ethanol into adult flies has been reported and used to show the involvement of GABA receptors in acute responses to alcohol (Dzitoyeva et al., 2003) (see section III.C.)

The most common way to expose flies to alcohol is to vaporize it and place the flies into a stream of defined ethanol saturation. This is most commonly done by bubbling an airstream through alcohol in a gas washing bottle, and combining this stream at predetermined flow rates with a second air stream that has been “washed” in pure water (Singh and Heberlein, 2000). In this way, flies will passively breathe in alcohol via their tracheal system in this continuous exposure paradigm. A variant of this exposure paradigm is to simply add a large drop of ethanol onto the cotton plug that keeps flies within their vial (Bhandari et al., 2009).

II. C. Measuring Alcohol-Induced Behaviors

To learn about the acute behavioral effect that alcohol has on flies, a number of assays have been developed that measure their behavioral response immediately following the (start) of the alcohol exposure. These assays measure the effect that alcohol has on four parameters: the flies’ position, posture, locomotion, and their consummatory behavior.

1. Positional Choice—One acute behavior that can be measured is whether flies are attracted to the smell of alcohol. One simple way to measure this is with an olfactory trap assay, where alcohol is placed in a vial sealed with a funnel (Reed, 1938). This allows flies to easily enter the vial, but few will escape. In this way the percentage of flies can be measured that is attracted by a given odor, or if two funneled traps are presented at the same time, a preference index can be calculated. An olfactory trap assay is commonly run over many hours, since the trap presents a small volume within a much larger receptacle. A more acute response to, and choice for or against alcohol can be measured in a Y- or T-maze. In this assay, the flies are motivated to run toward a choice point by their natural inclination for either negative geotaxis, or phototaxis. At the choice point they decide between one of two directions, and enter a vapor containing tube. This brief assay, generally run for around one minute, allows for the calculation of an attraction index, or when two odors are presented, for a preference index.

Positional choice is also measured in the context of oviposition preference and is measured in two ways (Joseph et al., 2009). First, adult females’ likelihood to spend time on ethanol-containing food can simply be filmed and compared to the time spent on a patch of control food. Second, their propensity to lay their eggs onto ethanol-containing food is measured by counting the number of eggs laid on this, versus control food.

2. Postural Control—When flies are exposed to a substantial dose of ethanol, they stop moving, start to lose postural control, and then fall over onto their backs unable to right themselves. This loss of postural control is the basis for the inebriometer. This apparatus was originally designed to measure the effects of volatile anaestethics on fly posture, but was subsequently applied to measure the response to ethanol vapor as well (Cohan and Graf, 1985). The inebriometer consists of a vertical column containing angled mesh baffles, into which typically 100 flies are introduced. Due to their natural inclination they tend to remain in the upper end of the cylinder, but as they are exposed to ethanol vapor, they start losing postural control, and drop down from one baffle onto the next, ultimately eluting from the column as a function of their sensitivity to ethanol. In this way, a mean elution time can be calculated for a given fly strain, representing a simple measure of that strains' sensitivity to ethanol. In addition, the inebriometer has also been used to selectively breed sensitive and resistant flies from a heterogeneous population (Cohan and Graf, 1985).

Because the inebriometer is a large and bulky apparatus, other ways to measure flies' loss of postural control have also been developed. Exposed flies can be challenged with a mechanical stimulus to ask how many have lost their righting reflex (Rothenfluh et al., 2006), or inspected visually to determine whether they are still standing, or lie sedated on their backs (Cowmeadow et al., 2005). Sometimes loss of locomotion activity has been used as a proxy for sedation (Park et al., 2000; Wen et al., 2005). However, it is worth noting that at an intermediate dose of alcohol the loss of locomotion activity precedes the loss of postural control by several minutes (A.R., unpublished observation), and certain fly strains seem to dissociate locomotion inactivity from loss of postural control (Rodan et al., 2002). Thus alcohol-induced inactivity should not be regarded as equivalent to sedation and loss of postural control.

If after a sedating dose of ethanol flies are left to recover in regular air, they will do so within five to 30 minutes. Since the time it takes to recover is longer if flies sedate early in a given exposure regimen, the recovery time has also been used as an indirect measure for fly strains' sensitivity to alcohol (Berger et al., 2004), similar to ethanol-induced sleep time measured in rodents. Since this recovery time is measured after ethanol exposure is finished, it may be that this measure will be more impacted by changes in ethanol pharmacokinetics, eg. mutations in *Adh*, catabolizing ethanol at a different rate. It is worth noting that *Adh* mutant flies showed only a minor effect on the time to ethanol-induced loss of posture as measured in the inebriometer as well as the internal alcohol concentration accrued during that time. Conversely, large differences in internal ethanol concentration were measured during recovery from the exposure (Singh and Heberlein, 2000).

3. Measures of Locomotion Activity—The natural tendency of flies for negative geotaxis is especially prominent after a mechanical startle, therefore the extent to which ethanol interferes with this reflexive response has been used as a measure of ethanol-induced akinesia, and/or loss of postural control (Bhandari et al., 2009). In this case, it is the absence of negative geotaxis locomotion that reflects the drug effect, and therefore this assay does not measure any activating effects that ethanol might have on spontaneous locomotion, as has been seen in many organisms. Early assays tackling ethanol-induced locomotion activation included manual counting of flies' line-crossing (Singh and Heberlein, 2000), and the inebri-actometer (Parr et al., 2001), a device that can measure up to 128 single flies, and how often they break an infrared beam as ethanol vapor is passed around them. Both of these assays indicated that flies show ethanol-induced hyperlocomotion, and the internal ethanol concentration at times of hyperactivity is about 20 mM (corresponding to a BAC of 0.09%), and 45 mM at the time of sedation (corresponding to 0.21%) (Singh and Heberlein, 2000). While both of these assays give some information regarding the activity of the flies, the temporal and spatial resolution of the locomotion analysis was not very detailed, and for

that reason a video based locomotion tracking system was designed (Wolf et al., 2002). In it, groups of 20 flies are exposed and their locomotion is tracked with an analysis software, which reveals 4 phases of the behavioral ethanol response: seconds after the start of the ethanol exposure, flies react by increasing their locomotion response. This is an olfactory-mediated startle response, since no measurable ethanol has accumulated inside flies yet, and since removal of the antennae, the primary olfactory sensory organ, abrogates this response (but not later hyperactivity) (Wolf et al., 2002). Within about one minute, the flies calm down again, most likely due to habituation to the startling ethanol odor (Cho et al., 2004). After a few more minutes, the flies begin to become hyperactive, and this is followed later by lack of movement, akinesia, and then sedation. Note that loss of postural control and akinesia cannot be distinguished with most video tracking setups, but flies can be visually inspected while they are being filmed, and the time to loss of righting can be determined simultaneously (Rothenfluh et al., 2006).

A careful examination of ethanol-induced locomotion response revealed that there is an ethanol concentration-dependent hyperactivity, with faster and earlier movement at higher alcohol concentrations. Locomotion occurs in bouts in flies, and as internal alcohol concentrations increased, individual flies showed faster locomotion during a bout, and longer bouts, until sedation started setting in, when those values both declined. Bout frequency on the other hand, did not change as a function of exposure time (Wolf et al., 2002).

4. Consummatory Behavior—As mentioned above, the CAFE assay allows for the precise measurement of ethanol-containing food consumed by flies over days. This can be combined with video tracking to allow for the measurement of both meal/drink size and their frequency.

II. D. Rapid Ethanol Tolerance

Repeat exposures to ethanol cause tolerance, i.e. a reduction in the effect of the same drug dose. Human alcoholics can have such high acquired alcohol tolerance that their blood alcohol levels would cause severe impairment, or even lethality in a naive, unexposed person (Urso et al., 1981). Since tolerance is one of the seven criteria for substance addiction (APA, 1994), it is thought to contribute to the development of alcoholism and is therefore of considerable interest to researchers. Different types of tolerance can be distinguished: metabolic tolerance refers to changes in ethanol pharmacokinetics due to changes in catabolism or drug distribution, while functional, or pharmacodynamic tolerance reflects changes in the central nervous system that lead to a decreased drug response upon re-exposure. Different ways of inducing tolerance are also differentiated: acute tolerance occurs during the alcohol experience, while rapid tolerance is induced after a single ethanol exposure that is followed by clearance of the drug from the organism’s system. Chronic tolerance is achieved by exposing the subject for multiple or prolonged times.

Adult Drosophila flies are a particularly useful organism to study functional alcohol tolerance. Unlike Drosophila larvae, they show no changes in enzymatic ADH levels when fed ethanol-containing food (Geer et al., 1988), and after the induction of rapid behavioral tolerance no changes in ethanol absorption or metabolism are observed (Berger et al., 2004; Cowmeadow et al., 2005; Scholz et al., 2000). Therefore, changes in ethanol-induced behavior are caused by changes in the nervous system (i.e., pharmacodynamic tolerance).

A single sedating exposure of flies in the inebriometer increased their mean elution time from 20 to 26 minutes when re-exposed 4 hours later (Scholz et al., 2000). This was also seen with a video tracking system, where pre-exposed flies showed a prolonged phase of hyperactivity, and delayed akinesia. The longer the first exposure, the more marked the

increase in mean elution time for the second exposure, up to a certain point, when tolerance reaches a plateau. This plateau is also seen with repeat sedating exposures given every 2 hours.

Maximum tolerance is seen 2 hours after the first exposure, after which time the amount of tolerance drops sharply, but remains at a steady lower level from 6 to 24 hours, still showing significant levels of tolerance a day later (Scholz et al., 2000). Inhibition of protein synthesis with cycloheximide did not change the extent of rapid tolerance four hours after the initial exposures, but it was not ascertained whether the lower level tolerance 24 hours after exposures is also unaffected (Berger et al., 2004).

The relationship between sensitivity or resistance to an acute alcohol exposure and tolerance is unclear. Berger and colleagues found that some acutely sensitive, normal, and resistant fly strains could all have lowered tolerance, while others could all have no effect on tolerance (Berger et al., 2008). In other words, the acute sensitivity of a fly strain does not predict its tolerance phenotype. In this context a word of caution is appropriate. If a given strain has an acute alcohol exposure phenotype, it is unclear what kind of exposure these flies should get to induce tolerance. Is it appropriate to expose sensitive and normal flies for the same duration, or do they need to be exposed until they each reach the same behavioral endpoint, i.e. just to sedation as in the inebriometer, but no longer? As mentioned above, the amount of tolerance induced is a variable depending on a number of parameters, including the dose of the first exposure, and unfortunately few studies investigating tolerance measure a tolerance dose response curve to ascertain that any tolerance defect found is consistently observed irrespective of initial exposure dose.

II. E. Chronic Tolerance

As discussed above, a single sedating exposure to alcohol induces rapid tolerance. In addition to such a regimen, a chronic exposure to low levels of alcohol (inducing neither hyperactivity nor sedation) for two days also causes flies to be more resistant to a high concentration ethanol challenge when compared to flies chronically exposed to air (Berger et al., 2004). The amount of tolerance induced by chronic exposure is slightly lower compared to a rapid induction (~30% sedation time increase compared to 50%), but it is longer lasting, and as opposed to rapid tolerance, significant chronic tolerance can still be observed 48 hours after the end of the ethanol pre-exposure. Given this long lasting effect of chronic preexposure, it is not surprising that the establishment of chronic tolerance required protein synthesis, while rapid tolerance did not. Furthermore, unlike rapid tolerance, chronic tolerance was not dependent on the biogenic amine octopamine, indicating that these two types of tolerance depend on different mechanisms.

II. F. Alcohol Preference

The common name of *Drosophila melanogaster* is vinegar, or pomace fly, and this implies that they would be found around rotting fruit with high likelihood. Ethanol levels present in fruit have been measured to be from 0.6% in ripe hanging fruit, to 4.5% in fallen rotten ones (Dudley, 2004). In addition, ripe fruit contain many other compounds and odors such as acetic acid (Chapman, 2003).

1. Female Oviposition Preference—Numerous studies have therefore investigated whether adult female vinegar flies would prefer to lay their eggs on food containing ethanol, given a choice. Richmond and Gerking showed that *D.melanogaster* strongly preferred to lay eggs on 9% ethanol-containing food, compared to regular food. This is in contrast to *D.willistoni*, which avoided laying on alcohol-containing food (Richmond and Gerking, 1979). When given a choice to lay on 15% ethanol-containing food however, normal

D.melanogaster flies preferred to lay on normal food. This was in contrast to flies kept on 12–18% ethanol containing food for 80 generations. These females preferred to lay on 15% ethanol-containing food (van Delden and Kamping, 1990). When flies were given a three-way choice between regular, 3% acetic acid, and 9% ethanol food, over 80% of the eggs were laid on the acetic acid food (Eisses, 1997). Thus overall it appears that ethanol is mildly attractive for females to lay their eggs on, but not nearly as much as other fruit-produced compounds such as acetic acid.

2. Adult Olfactory Responses to Ethanol—It has been appreciated for a long time that the smell of 5–15% ethanol solution is attractive to vinegar flies, while higher than 25% solutions are repellant. This is true for flies assayed over hours with a single odor-baited trap (Reed, 1938), or within 30 minutes in a odor versus solvent choice paradigm (Fuyama, 1976). Given the presence of ethanol in rotting fruit, it might be a significant attractant in the wild. However, compared to other odorants, the concentrations of ethanol need to be orders of magnitude higher to be attractive, compared to other odorants. Acetic acid for example is attractive at 0.1–0.5% (Fuyama, 1976), while other odorants are attractive at even lower concentrations.

3. Adult Preference and Alcohol Drinking—To ask whether adult vinegar flies like ethanol, Cadieu and colleagues (Cadieu et al., 1999) measured proboscis extension on 3% alcohol-containing, versus control food. They found that flies had an innate preference index (PI) of about 0.2 (where −1 is total avoidance, 0 is equal chance, and +1 is full preference), and this was increased to about 0.45 when they were kept on ethanol-food for 24 hours prior to the test. These results suggest that flies like the taste of ethanol, but the correlation between proboscis extension time and amount consumed was not established, thus it remained unclear whether flies prefer to actually consume alcohol. This question was tackled by Devineni and Heberlein (Devineni and Heberlein, 2009), and they found that flies' PI towards 15% ethanol-containing sucrose solution (over sucrose alone) would increase from 0.15 on the first, to 0.4 on the fifth day. When after establishing their drinking preference the flies underwent one or three days of forced abstinence, they immediately returned, or relapsed, to a high preference after having alcohol as a choice again. The authors also showed that flies would learn to overcome adversity to consume alcohol. When quinine was added to the sucrose, flies avoided it (PI= −0.1 to −0.2), and this avoidance remained constant over the 5 days tested. Adding ethanol to this sucrose/quinine solution did not alter aversion on the first day, but by day five the flies preferred this mix over sucrose alone (PI= 0.1 to 0.2). Thus overall these authors showed that flies would overcome adversity, or at least adverse taste, to consume alcohol, and that they would immediately relapse to pre-established high levels of drinking after a period of forced abstinence.

III. Genes and Biochemical Pathways Involved in Alcohol Responses

III. A. Forward Genetic Approaches

One of the first approaches to genetic differences in ethanol-induced behavior was to look at natural populations of Drosophila from the west coast of the US. Using the inebriometer, a significant trend was found that populations from more northern clines were more resistant to the sedating effects of ethanol (Cohan and Graf, 1985). Five subpopulations were then selectively bred for increased resistance in the inebriometer, and after 12 generations the mean sedation time increased from 14 to 28 minutes, indicating strong genetic contribution to acute ethanol sensitivity.

Singh and Heberlein (Singh and Heberlein, 2000) used three rounds of selection of a chemically randomly mutagenized population to set up individual resistant or sensitive fly strains with unique isolated X chromosomes. In this way they were able to isolate true-

breeding strains with mean inebriometer elution times ranging from 15 to 30 minutes (with wild type eluting in 20 minutes). Because of the semidominance of many of these mutations, and due to large effects by the genetic background of mapping strains, the mutant genes causing these phenotypes have never been isolated (Guarnieri and Heberlein, 2003). For that reason mutations with altered ethanol-resistance were then induced with transposable P-elements, which allow for quick determination of the genes affected, as the element's full sequence is known and sequence flanking the insertion site can easily be obtained. This approach has led to the isolation of mutations with altered acute ethanol sensitivity which affect cyclic AMP signaling (Moore et al., 1998), dynamic control of the actin cytoskeleton (Rothenfluh et al., 2006), or the map kinase/ERK pathway (Corl et al., 2005), as well as mutations affecting tolerance (Scholz et al., 2005) (see section III.C.)

III. B. Genomic Approaches

Numerous studies have taken advantage of genome-wide approaches that can be performed with microarray technology. Most of those studies compared gene expression levels before and after alcohol exposure (Kong et al., 2010a), sometimes including repeat exposures resulting in the development of tolerance (Morozova et al., 2006; Urizar et al., 2007). It is worth noting that in these three studies, only 14% of 1669 significantly changed transcripts were found in at least two of the three studies (Kong et al., 2010a). This may be due to differences in the exact design of the ethanol exposure, or the data capture and analysis, or in the respective wild type flies used for exposure. Nevertheless, numerous functional groups of genes were repeatedly overrepresented, and many candidate genes were found that had mutations with altered alcohol-induced behaviors.

In two studies, Morozova, Mackay and colleagues combined genetic variation with genomic approaches to find genes and pathways involved in alcohol responses. The first study used wild-type fly strains selected over 35 generations for sensitivity or resistance to ethanol to assay transcriptional profiles (Morozova et al., 2007). Currently, there is a greater than 90% chance that for any given gene a mutant strain exists that can be easily obtained from public stock centers. For that reason, many genes that show altered expression levels in the above transcription profiling approaches can quickly be tested for any behavioral ethanol phenotypes. Morozova and colleagues, for example, tested 37 candidate gene mutations of genes differentially expressed in sensitive versus resistant strains, and found 32 of those had significantly different sensitivity to an acute ethanol exposure. Thus in flies, these approaches can quickly lead to the isolation of new genes and mutations that are behaviorally relevant.

In an exciting new approach, Morozova and colleagues assayed 40 isogenic lines derived from wild populations for their alcohol sensitivity (Morozova et al., 2009). The interesting thing about those 40 strains is that they are all fully sequenced, and transcriptional profiles are available for all 40. This allowed for the correlation between ethanol-sensitivity, genomic polymorphisms, and mRNA abundance, including the identification of potential cis-regulatory elements shared between co-regulated genes. In addition, this approach can highlight the trans-regulators which act upon a given set of co-regulated genes.

III. C. Functional Pathways in Alcohol-Induced Behavior

1. Overrepresentation in transcriptional profiling—Three studies have investigated changes in transcript levels as a result of acute alcohol exposures. While a surprisingly small number of the ethanol-regulated transcripts was found in all three studies (2%), or even two of the three (14%), some gene ontology (GO) annotations were significantly overrepresented in the ethanol-regulated transcripts in all three studies. These categories were regulators of signal transduction, transcription factors and metabolic enzymes. Indeed, of the 25 genes

found in all three studies as similarly ethanol-regulated, twelve are annotated with the GO terms ‘metabolic and biosynthesis processes’, and three are involved in serine biosynthesis (Kong et al., 2010a). Yet no mutations of these genes have been tested for their behavioral ethanol phenotype.

Based on transcriptional profiling, a number of mutations in intermediary metabolism and fatty acid biosynthesis enzymes were found to alter acute ethanol sensitivity, including pyruvate dehydrogenase kinase, pyruvate carboxylase, and malic enzyme (Morozova et al., 2006). Genes associated with pyruvate metabolism were also found differentially expressed in alcohol preferring and non-preferring mice (Mulligan et al., 2006). Malic enzyme serves as a link between the glycolytic pathway, the tri-carboxylic acid cycle, and fatty acid synthesis, allowing for conversion of excess ethanol-derived energy into lipid biosynthesis. Indeed, alcohol-induced fatty acid synthesis is well documented in alcoholics (Lieber, 2004). Interestingly, a polymorphism in human malic enzyme associated with cocktail drinking was then found in the Framingham Offspring cohort, albeit with a small effect (Morozova et al., 2009).

It is noteworthy that many genes that affect the response to alcohol (see next sections) showed no change in these transcriptome analyses (Morozova et al., 2006), indicating that this approach only captures a subset of the genes involved in, and relevant for mediating behavioral alcohol responses.

2. Stress and Ethanol Tolerance—Many genes involved in stress responses like heat shock proteins, cytochrome P450 proteins and glutathione transferases are upregulated after an acute ethanol exposure. Indeed, Kong and colleagues (Kong et al., 2010a) found that 5 of the 20 genes with the highest ethanol-induced induction, and 9 of the 21 *Hsp* genes overall had significantly increased expression levels after ethanol exposure.

The *hangover* mutation was found in a forward genetic screen to greatly reduce rapid tolerance. When flies were stressed by a 30 minute exposure to a 37°C heat shock, instead of a sedating ethanol pre-exposure, they developed cross-tolerance to ethanol exposure, similar in magnitude as if they were pre-exposed to ethanol. *hangover* mutants showed a reduction in this stress-induced cross-tolerance, while the rapid tolerance mutants *Tbh* (see next section) were completely normal. This suggests that the development of ethanol tolerance in flies is composed of a Tbh- and octopamine-dependent, but stress-independent component, as well as a *hangover*-dependent stress component. Indeed, when *hangover* mutants were exposed to oxidative stress, they were more susceptible than wild-type flies, arguing that *hangover* is a gene required for normal stress responses (Scholz et al., 2005). Interestingly, there is another connection between stress- and ethanol-resistance. Flies that were selectively bred for alcohol resistance also showed increased resistance to such stressors as heat, desiccation and other chemicals (Cohan and Hoffmann, 1986), and flies selected to tolerate high levels of desiccation also co-selected resistance to ethanol (Hoffmann and Parsons, 1989).

It is unclear what the exact molecular function of the *hangover* gene is, but it encodes a zinc-finger transcription factor, and is involved in the regulation of synaptic growth in the larval neuromuscular junction (Schwenkert et al., 2008). It is currently not known whether central synapses are also affected in *hangover* mutants. The human *hangover* ortholog is called *ZNF699*, and polymorphisms in this gene were found associated with alcoholic cases in a study of Irish sibling pairs. In addition, one risk haplotype was associated with decreased gene expression in the dorsolateral prefrontal cortex in postmortem cases (Riley et al., 2006).

3. Octopamine, dopamine and cyclic AMP signaling—One of the first mutations with altered ethanol tolerance was found in the *Tbh* gene, encoding tyramine β-hydroxylase. This enzyme converts tyramine to octopamine, a biogenic amine thought to have similar functions in invertebrates as norepinephrine does in mammals (Monastirioti, 1999). The latter has been implicated in the development of ethanol tolerance in rats (Ritzmann and Tabakoff, 1976). Flies with a mutation in *Tbh* showed a reduction of 50–60% in the development of tolerance 4 hrs after a single sedating alcohol exposure.

Dopamine is an important neurotransmitter in the mammalian reward system, and is strongly implicated in the development of addictions. Bainton and colleagues showed that inhibiting tyrosine hydroxylase (TH), the rate limiting synthesis enzyme for dopamine, resulted in decreased locomotor-hyperactivation by ethanol, and this effect could be rescued by feeding flies L-dopa, the product of TH activity and immediate precursor of dopamine (Bainton et al., 2000). The different dopamine receptors couple to adenyl cyclase and increase or decrease cyclic AMP (cAMP) levels and protein kinase A (PKA) activity, depending on the receptor subtype. The first evidence that cAMP signaling was involved in Drosophila ethanol responses came from a forward genetic transposon screen that yielded the sensitive mutant *cheapdate* (Moore et al., 1998). This mutation turned out to be an allele of *amnesiac*, a gene which is important in learning and memory, and encodes the fly ortholog of pituitary adenylate cyclase-activating peptide, PACAP. This study also found that mutations in genes resulting in reduced cAMP signaling, such as mutations in genes encoding adenylate cyclase, or PKA cause increased ethanol-sensitivity. Activation of the cAMP pathway by forskolin rescued the sensitivity caused by *cheapdate*. In addition, a mutation in the PKA regulatory subunit, thought to increase cAMP signaling, caused resistance to the sedating effects of ethanol (Park et al., 2000). Olfactory learning and memory critically depends on proper cAMP signaling and the genes encoding adenylate cyclase (*rutabaga*), or cAMP phosphodiesterase (*dunce*, hence the gene names). Since both behavioral alcohol responses, and associative learning depend on cAMP, it is therefore not surprising that in a substantial collection of learning mutants, many had ethanol sedation or tolerance phenotypes as well (Berger et al., 2008).

Consistent with the above results, Rodan and colleagues found that inhibition of PKA by globally expressing a UAS-PKA-inhibitor construct caused sensitivity to ethanol-induced sedation (Rodan et al., 2002). However, when the inhibitor was expressed only in certain subsets of the brain, it caused resistance (Rodan et al., 2002), while in others it caused sensitivity (Corl et al., 2005). This indicates that different neurons and circuits in the fly brain utilize cAMP signaling to mediate either resistance or sensitivity to ethanol.

2. Neuropeptide signaling—The neurons that mediated ethanol sensitivity after PKA inhibition contained peptidergic neurosecretory cells, amongst other peptides. Demonstrating that insulin-like peptide is involved in behavioral ethanol responses was the finding that insulin receptor mutants also caused sensitivity, as did inhibition of PI3K, or overexpression of constitutively-active transcription factor FOXO, two members of the downstream insulin signaling cascade.

Another neuropeptide involved in Drosophila ethanol responses is NPF, the invertebrate ortholog of NPY. Overexpression of NPF caused sensitivity to ethanol-induced sedation, while ablating NPF-expressing neurons with diphteria toxin caused resistance to ethanol (Wen et al., 2005). Mice lacking NPY displayed increased resistance to ethanol sedation, and increased ethanol consumption, while overexpression of NPY caused the opposite phenotypes (Thiele et al., 1998).

3. Synaptic transmission and ion channels—A candidate gene that came from an ethanol responsive transcript analysis was the post synaptic scaffolding and signaling molecule homer. Transcript levels of homer decreased after ethanol exposure, and mutations in the gene caused sensitivity (Urizar et al., 2007). Interestingly, homer knock-out mice also showed sensitivity to ethanol, and decreased voluntary drinking and ethanol-induced place preference (Szumlinski et al., 2005).

In vertebrates, the $GABA_B$ receptor is involved in ethanol-mediated behavior (Maccioni et al., 2009). This metabotropic receptor is conserved in flies, and knock-down of the receptor with intra-abdominal small interfering RNA caused flies to show reduced sedation upon ethanol injection. Similarly, co-injection of ethanol and the $GABA_B$ antagonist CGP 54626 also decreased sedation. Pretreatment with the agonist 3-APMPA on the other hand, abolished the development of rapid ethanol tolerance (Dzitoyeva et al., 2003).

The presynaptic vesicle scaffolding protein synapsin has also been implicated in ethanol responses. Flies lacking their only synapsin gene showed an increase in rapid ethanol tolerance (Godenschwege et al., 2004). Interestingly, mammalian synapsins I and II showed increased phosphorylation upon an exposure of ethanol, which was adenylate cyclase, and thus presumably PKA-dependent (Conti et al., 2009).

The pore-forming subunits of the CA^{2+}-activated BK potassium channel are encoded by the *slowpoke* gene in flies. This channel is highly conserved between worms, flies, and vertebrates and its activity is potentiated by direct ethanol binding (Treistman and Martin, 2009). Worms with mutations in the BK channel showed resistance to ethanol (Davies et al., 2003). In flies, the *slo* gene has five different tissue-specific promoters (Bohm et al., 2000), and a nervous system-specific mutant abolished ethanol-induced rapid tolerance (Cowmeadow et al., 2005). Interestingly, over-expressing the CNS-specific isoform of *slo* induced tolerance (Cowmeadow et al., 2006), and *slo* was differentially expressed in selectively bred resistant versus sensitive strains (Morozova et al., 2007).

4. Transcription factors and epigenetics—The anaesthetic benzyl alcohol also induces rapid tolerance in flies (Ghezzi et al., 2004), and benzyl alcohol-mediated sedation induced *slo* expression via the transcription factor CREB2 (Wang et al., 2007). Flies without CREB2 were unable to acquire tolerance to benzyl alcohol, and did not upregulate *slo* expression after alcohol exposure (Wang et al., 2009). In addition, subsets of the *slo* promoter showed altered histone H4 acetylation patterns after benzyl alcohol-induced sedation, as demonstrated with chromatin immunoprecipitation, and the histone de-acetylase inhibitor sodium butyrate induced *slo* transcripts, and resistance to benzyl alcohol sedation (Wang et al., 2007). Considerable evidence indicates that epigenetic regulation is an important contributor to mammalian addiction as well (reviewed in Renthal and Nestler, 2008), including responses to ethanol (Pandey et al., 2008).

5. Cytoskeleton—In a meta-analysis of transcript changes between mouse strains with different alcohol consumption patterns, the two most overrepresented GO categories were ‘regulation of the actin cytoskeleton’, and the MAP kinase signaling pathway (Mulligan et al., 2006).

It has been known for a while that an acute ethanol exposure of cultured cells results in rearrangement of the actin cytoskeleton (Allansson et al., 2001), and in cultured rat hippocampal cells, chronic ethanol caused an increase in dendritic spine number and levels of filamentous actin (Carpenter-Hyland and Chandler, 2006). Exposing cultured cells to alcohol also led to a reorganization of their actin cytoskeleton. In astrocytes this reorganization is mediated by an ethanol-induced decrease of GTP-loaded RhoA, which can

be reversed by exogenous RhoA activation via lysophosphatic acid (Martinez et al., 2007). Does this effect of ethanol on the actin cytoskeleton have any significance in alcohol-related behaviors, or is it merely a reflection of its cytotoxicity? A mutation in the fly *RhoGAP18B* gene caused resistance to the sedating effects of ethanol (Rothenfluh et al., 2006). This gene encodes a GTPase activating protein that likely exerts its effect on ethanol-induced sedation via the Rho or Rac GTPases, key regulators of the actin cytoskeleton. Interestingly, *RhoGAP18B* also encodes a smaller isoform, which specifically affected ethanol-induced hyperactivation, and not sedation. Thus proper actin regulation is important for both ethanol-induced hyperactivation, as well as sedation. Coincident with the discovery of RhoGAP18B in ethanol responses, Offenhäuser and colleagues found that a genetic knock-out of an actin-capping protein, Eps8, led to mice that are resistant to ethanol's sedative effects and that also drank more in a two bottle choice paradigm (Offenhauser et al., 2006). Primary cell cultures of normal cerebellar granule cells lost filamentous F-actin and decreased NMDA receptor current when exposed to ethanol, and these two ethanol-responses were lost in Eps8 KO mice, indicating a strong correlation between the dynamics of the actin cytoskeleton and drinking behavior.

Some other mutations in fly genes involved in actin dynamics have alcohol behavior phenotypes. Berger and colleagues (Berger et al., 2008) found that mutations in the actin nucleator *formin3* caused increased acute ethanol sensitivity, and reduced rapid tolerance, while a mutation in the actin binding filamin protein cheerio also caused increased acute sensitivity. On the other hand, mutations in the RAB11FIP3/4 encoding gene *nuf*, which is involved in vesicle trafficking and actin organization, caused resistance to acute ethanol sedation (Morozova et al., 2007). Lastly, the mouse *addicsin* gene encodes ARL6IP5 which is involved in the development of morphine tolerance and dependence (Ikemoto et al., 2002). RNAi-mediated knock-down of the fly ortholog caused a decrease, while overexpression caused an increase in rapid ethanol tolerance (Li et al., 2008a). The molecular function of ARL6IP5 is not exactly known, but it is microtubule-associated, and involved in F-actin rearrangement and MAP kinase activation (Chen and Olsen, 2007). It has also been shown to regulate the excitatory amino acid carrier-1 (Watabe et al., 2007), and inhibit the trafficking molecule Rab1 (Maier et al., 2009).

6. Growth factors—As mentioned above, the MAP kinase signaling pathway was overrepresented in a transcriptome meta-analysis between dinking, and non-drinking mice (Mulligan et al., 2006). In addition, a meta-analysis of over 1000 publications about various drug addictions also highlighted this pathway as significantly over-represented (Li et al., 2008b). In flies, an unbiased genetic screen yielded mutations in the Ste20-family kinase gene *happy hour,* that caused resistance to ethanol-induced sedation (Corl et al., 2009). This kinase is a negative regulator of the EGF receptor pathway, and loss-of-function mutations in pathway members, like the receptor ligand Spitz, or the Spitz-activating protease rhomboid, caused sensitivity, while activating the pathway by overexpressing the receptor or constitutive active ERK kinase caused resistance (Corl et al., 2009). This study also showed that the EGF receptor inhibitor erlotinib caused ethanol-sensitivity in flies and mice, and reduced voluntary ethanol intake in rats for 24 hours after inhibitor administration.

7. Cell adhesion—Numerous cell adhesion molecules have been found associated with addiction disorders, including alcoholism (Li et al., 2009). In flies, the best studied group of cell adhesion molecules and their effects on ethanol-induced behaviors is the integrin family of cell adhesion and signaling molecules. Mutations in the β-integrin encoding genes *mys* (Bhandari et al., 2009) and *βint-v* (Morozova et al., 2007), as well as the a-integrin encoding gene *scab* (Bhandari et al., 2009; Kong et al., 2010a) all caused acute ethanol sensitivity, and both *mys* and *scab* mutants showed increased rapid ethanol tolerance (Bhandari et al., 2009). The NCAM-encoding gene *fasII* was also required for normal acute resistance to alcohol,

and for normal olfactory learning (Cheng et al., 2001). Mutations in the Notch-regulated cell adhesion gene *klingon* caused both defects in long-term memory (Matsuno et al., 2009) and acute ethanol-sensitivity, and decreased chronic and rapid tolerance (Berger et al., 2008), again highlighting the molecular overlap between learning and memory, and behavioral ethanol responses.

V. Neural Circuits Involved in Ethanol Responses

In the mammalian brain, a number of brain regions and circuits are well known to play key roles in addiction, including alcohol drinking. These include the mesolimbic dopamine pathway, which includes dopaminergic neurons in the ventral tegmental area that mediate a reinforcement signal, and their targets in the forebrain, notably the nucleus accumbens. Are there functionally equivalent circuits in the fly brain? And which other regions in the fly brain are involved in behavioral responses to alcohol?

Numerous studies have shown that an alcohol-induced behavioral phenotype could be rescued by expressing the mutated gene's wild-type cDNA specifically in the CNS. This was true for Ste20-family kinase encoded by *happy hour* (Corl et al., 2009), the tolerance gene *hangover* (Scholz et al., 2005), or the postsynaptic protein homer (Urizar et al., 2007). The *homer* gene is more specifically required in the ellipsoid body of the brain, a region that is also involved in rapid tolerance (Scholz et al., 2000). This brain region is known to be important in the control of locomotion behavior, and is also involved in GABA-mediated spatial working-type memory (Neuser et al., 2008). Other neurochemical systems involved in behavioral ethanol responses mentioned above include dopamniergic and octopaminergic neurons, as well as NPF- or insulin-like peptide producing neurosecretary cells.

Neurons expressing the amnesiac peptide project to the mushroom bodies, which are crucial for amnesiac-dependent learning and memory (Waddell et al., 2000). While the *amnesiac* allele *cheapdate* leads to ethanol-sensitivity (Moore et al., 1998), the mushroom bodies are not required for normal ethanol-induced sedation (Rodan et al., 2002). Thus the PACAP ortholog amnesiac mediates normal ethanol sedation via different neurons. Rodan and colleagues (Rodan et al., 2002) assayed 64 Gal4-expressing lines to test which neurons required PKA signaling to mediate normal ethanol-sedation. The three Gal4 lines that showed significant ethanol-resistance when expressing a PKA inhibitor did unfortunately not show significant overlap in the expressing neurons. These studies did, however, indicate five things: first, the Gal4-binary system is a viable approach to learn about the anatomical requirements of ethanol-induced behaviors. Second, multiple types of neurons, and possibly circuits are involved in mediating ethanol-induced sedation. This finding is highlighted by the fact that PKA inhibition in insulin-like peptide-expressing neurosecretory cells caused sensitivity to ethanol (Corl et al., 2005), unlike the three Gal4 lines mentioned above, which caused resistance. Third, the neurons involved in ethanol-induced sedation, are not necessarily the same neurons mediating ethanol-induced hyperactivity. Indeed, none of these three Gal4 lines causing ethanol-resistance altered the amplitude of ethanol-induced hyperactivation (Rodan et al., 2002), which was reduced in flies that had reduced dopamine levels (Bainton et al., 2000). Fourth, while there is a substantial overlap in the molecules required for behavioral alcohol-responses and for associative memory, the neural substrates of these behaviors seem distinct. And fifth, even though hundreds of differentially expressing Gal4 lines are available in flies, there is a need for both more spatially restricted, and better characterized lines. As mentioned in the introduction, this very problem is currently being tackled on a large scale (Pfeiffer et al., 2008), and it will be fascinating to see whether the functional organization of the fly brain bears any resemblance to the mammalian brain.

V. Conclusions

A variety of approaches and assays have been used to isolate many genes that are involved in the behavioral responses to alcohol. Many of these genes are also involved in mammalian ethanol behaviors, including allelic associations in human alcoholic cohorts. Therefore the fly has proven to be an exceptionally useful model organism to find the molecular components, and therefore potential risk factors and therapeutic targets, which mediate these behaviors. The genes mentioned in this review, affecting behavioral responses to alcohol in the fly fall into a many diverse functional groups. Since addiction hijacks endogenous reward and reinforcement-learning pathways, it is not surprising that many of the mentioned genes would be involved in synaptic plasticity, learning and memory. The accumulated evidence so far indicates that there are no single gene mutations that by themselves are sufficient to cause addiction. Rather, many genetic variants are likely involved that each contribute a small risk to the development of alcohol abuse. For this reason the fly is an excellent model organism to identify many of those genes, and because of its experimental accessibility, it is possible to mechanistically dissect the function of those genes and their associated molecular partners. With the recent development of a reliable alcohol drinking and preference assay (Devineni and Heberlein, 2009), together with the development of new tools allowing the precise spatial (Pfeiffer et al., 2008), and dynamic (Pulver et al., 2009) control of small groups of neurons, Drosophila is poised to generate yet more insights into the basic biology, and molecular and functional mechanisms that underlie the transition from alcohol exposure, to excessive consumption of this widely abused drug.

References

Adams MD, Celniker SE, Holt RA, Evans CA, Gocayne JD, Amanatides PG, Scherer SE, Li PW, Hoskins RA, Galle RF, et al. The genome sequence of Drosophila melanogaster. Science (New York, NY). 2000; 287:2185–2195.

Allansson L, Khatibi S, Olsson T, Hansson E. Acute ethanol exposure induces [Ca2+]i transients, cell swelling and transformation of actin cytoskeleton in astroglial primary cultures. Journal of neurochemistry. 2001; 76:472–479. [PubMed: 11208910]

APA. American Psychiatric Association: Diagnostic and Statistical Manual of Mental Disorders IV. Washington, DC: American Psychiatric Association; 1994.

Bainton R, Tsai LT, Singh CM, Moore MS, Neckameyer WS, Heberlein U. Dopamine modulates acute responses to cocaine, nicotine and ethanol in Drosophila. Current Biology. 2000; 10:187–194. [PubMed: 10704411]

Berger KH, Heberlein U, Moore MS. Rapid and Chronic: Two Distinct Forms of Ethanol Tolerance in Drosophila. Alcoholism: Clinical and Experimental Research. 2004; 28:1469–1480.

Berger KH, Kong EC, Dubnau J, Tully T, Moore MS, Heberlein U. Ethanol sensitivity and tolerance in long-term memory mutants of Drosophila melanogaster. Alcoholism: Clinical and Experimental Research. 2008; 32:895–908.

Bhandari P, Kendler KS, Bettinger JC, Davies AG, Grotewiel MS. An Assay for Evoked Locomotor Behavior in Drosophila Reveals a Role for Integrins in Ethanol Sensitivity and Rapid Ethanol Tolerance. Alcoholism: Clinical and Experimental Research. 2009

Bohm RA, Wang B, Brenner R, Atkinson NS. Transcriptional control of Ca(2+)-activated K(+) channel expression: identification of a second, evolutionarily conserved, neuronal promoter. Journal of Experimental Biology. 2000; 203:693–704. [PubMed: 10648211]

Brand AH, Perrimon N. Targeted gene expression as a means of altering cell fates and generating dominant phenotypes. Development (Cambridge, England). 1993; 118:401–415.

Cadieu N, Cadieu J, El Ghadraoui L, Grimal A, Lamboeuf Y. Conditioning to ethanol in the fruit fly-a study using an inhibitor of ADH. Journal of Insect Physiology. 1999; 45:579–586. [PubMed: 12770343]

Carpenter-Hyland EP, Chandler LJ. Homeostatic plasticity during alcohol exposure promotes enlargement of dendritic spines. European Journal of Neuroscience. 2006; 24:3496–3506. [PubMed: 17229098]

Chapman RF. Contact chemoreception in feeding by phytophagous insects. Annual review of entomology. 2003; 48:455–484.

Chen ZW, Olsen RW. GABAA receptor associated proteins: a key factor regulating GABAA receptor function. Journal of neurochemistry. 2007; 100:279–294. [PubMed: 17083446]

Cheng Y, Endo K, Wu K, Rodan AR, Heberlein U, Davis RL. Drosophila fasciclinII is required for the formation of odor memories and for normal sensitivity to alcohol. Cell. 2001; 105:757–768. [PubMed: 11440718]

Chien S, Reiter LT, Bier E, Gribskov M. Homophila: human disease gene cognates in Drosophila. Nucleic Acids Research. 2002; 30:149–151. [PubMed: 11752278]

Cho W, Heberlein U, Wolf FW. Habituation of an odorant-induced startle response in Drosophila. Genes, Brain and Behavior. 2004; 3:127–137.

Cohan FM, Graf J. Latitudinal Cline in Drosophila melanogaster for Knockdown Resistance to Ethanol Fumes and for Rates of Response to Selection for Further Resistance. Evolution. 1985; 39:278–293.

Cohan FM, Hoffmann AA. Genetic divergence under uniform selection. II. Different responses to selection for knockdown resistance to ethanol among Drosophila melanogaster populations and their replicate lines. Genetics. 1986; 114:145–164. [PubMed: 3095180]

Collins B, Blau J. Even a stopped clock tells the right time twice a day: circadian timekeeping in Drosophila. Pflüogers Archiv: European journal of physiology. 2007; 454:857–867.

Conti AC, Maas JW, Moulder KL, Jiang X, Dave BA, Mennerick S, Muglia LJ. Adenylyl cyclases 1 and 8 initiate a presynaptic homeostatic response to ethanol treatment. PLoS ONE. 2009; 4:e5697. [PubMed: 19479030]

Corl A, Rodan AR, Heberlein U. Insulin signaling in the nervous system regulates ethanol intoxication in Drosophila melanogaster. Nature Neuroscience. 2005; 8:18–19.

Corl AB, Berger KH, Ophir-Shohat G, Gesch J, Simms JA, Bartlett SE, Heberlein U. Happyhour, a Ste20 Family Kinase, Implicates EGFR Signaling in Ethanol-Induced Behaviors. Cell. 2009

Cowmeadow RB, Krishnan HR, Atkinson NS. The slowpoke gene is necessary for rapid ethanol tolerance in Drosophila. Alcoholism: Clinical and Experimental Research. 2005; 29:1777–1786.

Cowmeadow RB, Krishnan HR, Ghezzi A, Al'Hasan YM, Wang YZ, Atkinson NS. Ethanol tolerance caused by slowpoke induction in Drosophila. Alcoholism: Clinical and Experimental Research. 2006; 30:745–753.

David JR, Bocquet C, Arens MF, Fouillet P. Biological role of alcohol dehydrogenase in the tolerance of Drosophila melanogaster to aliphatic alochols: utilization of an ADH-null mutant. Biochemical genetics. 1976; 14:989–997. [PubMed: 828053]

Davies AG, Pierce-Shimomura JT, Kim H, VanHoven MK, Thiele TR, Bonci A, Bargmann CI, McIntire SL. A central role of the BK potassium channel in behavioral responses to ethanol in C. elegans. Cell. 2003; 115:655–666. [PubMed: 14675531]

Devineni AV, Heberlein U. Preferential ethanol consumption in Drosophila models features of addiction. Current Biology. 2009; 19:2126–2132. [PubMed: 20005106]

Dietzl G, Chen D, Schnorrer F, Su KC, Barinova Y, Fellner M, Gasser B, Kinsey K, Oppel S, Scheiblauer S, et al. A genome-wide transgenic RNAi library for conditional gene inactivation in Drosophila. Nature. 2007; 448:151–156. [PubMed: 17625558]

Dudley R. Ethanol, Fruit Ripening, and the Historical Origins of Human Alcoholism in Primate Frugivory. Integr Comp Biol. 2004; 44:315–323. [PubMed: 21676715]

Dzitoyeva S, Dimitrijevic N, Manev H. Gamma-aminobutyric acid B receptor 1 mediates behavior-impairing actions of alcohol in Drosophila: adult RNA interference and pharmacological evidence. Proceedings of the National Academy of Sciences of the United States of America. 2003; 100:5485–5490. [PubMed: 12692303]

Eisses KT. The influence of 2-propanol and acetone on oviposition rate and oviposition site preference for acetic acid and ethanol of Drosophila melanogaster. Behavior genetics. 1997; 27:171–180. [PubMed: 9210788]

Fuyama Y. Behavior genetics of olfactory responses in Drosophila. I. Olfactometry and strain differences in Drosophila melanogaster. Behavior genetics. 1976; 6:407–420. [PubMed: 825093]

Geer BW, McKechnie SW, Bentley MM, Oakeshott JG, Quinn EM, Langevin ML. Induction of alcohol dehydrogenase by ethanol in Drosophila melanogaster. The Journal of nutrition. 1988; 118:398–407. [PubMed: 3127555]

Gehring WJ, Kloter U, Suga H. Evolution of the Hox gene complex from an evolutionary ground state. Current topics in developmental biology. 2009; 88:35–61. [PubMed: 19651301]

Ghezzi A, Al-Hasan YM, Larios LE, Bohm RA, Atkinson NS. slo K(+) channel gene regulation mediates rapid drug tolerance. Proceedings of the National Academy of Sciences of the United States of America. 2004; 101:17276–17281. [PubMed: 15569939]

Godenschwege TA, Reisch D, Diegelmann S, Eberle K, Funk N, Heisenberg M, Hoppe V, Hoppe J, Klagges BR, Martin JR, et al. Flies lacking all synapsins are unexpectedly healthy but are impaired in complex behaviour. European Journal of Neuroscience. 2004; 20:611–622. [PubMed: 15255973]

Guarnieri DJ, Heberlein U. Drosophila melanogaster, a genetic model system for alcohol research. International review of neurobiology. 2003; 54:199–228. [PubMed: 12785288]

Hoffmann AA, Parsons PA. Selection for increased desiccation resistance in Drosophila melanogaster: additive genetic control and correlated responses for other stresses. Genetics. 1989; 122:837–845. [PubMed: 2503423]

Ikemoto MJ, Inoue K, Akiduki S, Osugi T, Imamura T, Ishida N, Ohtomi M. Identification of addicsin/GTRAP3-18 as a chronic morphine-augmented gene in amygdala. Neuroreport. 2002; 13:2079–2084. [PubMed: 12438930]

Ja W, Carvalho G, Mak E, De La Rosa N, Fang A, Liong J, Brummel T, Benzer S. Prandiology of Drosophila and the CAFE assay. Proceedings of the National Academy of Sciences of the United States of America. 2007; 104:8253–8256. [PubMed: 17494737]

Joseph RM, Devineni AV, King IF, Heberlein U. Oviposition preference for and positional avoidance of acetic acid provide a model for competing behavioral drives in Drosophila. Proceedings of the National Academy of Sciences of the United States of America. 2009; 106:11352–11357. [PubMed: 19541615]

Kitamoto T. Conditional modification of behavior in Drosophila by targeted expression of a temperature-sensitive shibire allele in defined neurons. Journal of neurobiology. 2001; 47:81–92. [PubMed: 11291099]

Kong EC, Allouche L, Chapot PA, Vranizan K, Moore MS, Heberlein U, Wolf FW. Ethanol-Regulated Genes That Contribute to Ethanol Sensitivity and Rapid Tolerance in Drosophila. Alcoholism: Clinical and Experimental Research. 2010a; 34:1–14.

Kong EC, Woo K, Li H, Lebetsky T, Mayer N, Sniffen MR, Heberlein U, Bainton RJ, Hirsh J, Wolf FW. A Pair of Dopamine Neurons Target the D1-Like Dopamine Receptor DopR in the Central Complex to Promote Ethanol-Stimulated Locomotion in *Drosophila*. PLoS ONE. 2010b; 5:e9954. [PubMed: 20376353]

LaFerriere H, Guarnieri DJ, Sitaraman D, Diegelman S, Heberlein U, Zars T. Genetic dissociation of ethanol sensitivity and memory formation in *Drosohila melanogaster*. Genetics. 2008; 178:1095–1902.

Li C, Zhao X, Cao X, Chu D, Chen J, Zhou J. The Drosophila homolog of jwa is required for ethanol tolerance. Alcohol and Alcoholism. 2008a; 43:529–536. [PubMed: 18503079]

Li CY, Liu QR, Zhang PW, Li XM, Wei L, Uhl GR. OKCAM: an ontology-based, human-centered knowledgebase for cell adhesion molecules. Nucleic Acids Research. 2009; 37:D251–260. [PubMed: 18790807]

Li CY, Mao X, Wei L. Genes and (common) pathways underlying drug addiction. PLoS computational biology. 2008b; 4:e2. [PubMed: 18179280]

Lieber CS. Alcoholic fatty liver: its pathogenesis and mechanism of progression to inflammation and fibrosis. Alcohol. 2004; 34:9–19. [PubMed: 15670660]

Maccioni P, Colombo G. Role of the GABA(B) receptor in alcohol-seeking and drinking behavior. Alcohol. 2009; 43:555–558. [PubMed: 19913200]

Maggert KA, Gong WJ, Golic KG. Methods for homologous recombination in Drosophila. Methods in molecular biology (Clifton, NJ). 2008; 420:155–174.

Maier S, Reiterer V, Ruggiero AM, Rothstein JD, Thomas S, Dahm R, Sitte HH, Farhan H. GTRAP3-18 serves as a negative regulator of Rab1 in protein transport and neuronal differentiation. Journal of cellular and molecular medicine. 2009; 13:114–124. [PubMed: 18363836]

Martinez S, Lazaro-Dieguez F, Selva J, Calvo F, Piqueras J, Crespo P, Claro E, Egea G. Lysophosphatidic acid rescues RhoA activation and phosphoinositides levels in astrocytes exposed to ethanol. Journal of neurochemistry. 2007; 102:1044–1052. [PubMed: 17442046]

Matsuno M, Horiuchi J, Tully T, Saitoe M. The Drosophila cell adhesion molecule klingon is required for long-term memory formation and is regulated by Notch. Proceedings of the National Academy of Sciences of the United States of America. 2009; 106:310–315. [PubMed: 19104051]

Matthews KA, Kaufman T, Gelbart WM. Research resources for Drosophila: the expanding universe. Nature Reviews Genetics. 2005; 6:179–193.

Monastirioti M. Biogenic amine systems in the fruit fly Drosophila melanogaster. Microscopy research and technique. 1999; 45:106–121. [PubMed: 10332728]

Moore MS, DeZazzo J, Luk AY, Tully T, Singh CM, Heberlein U. Ethanol intoxication in Drosophila: Genetic and pharmacological evidence for regulation by the cAMP signaling pathway. Cell. 1998; 93:997–1007. [PubMed: 9635429]

Morozova TV, Anholt RR, Mackay TFC. Transcriptional response to alcohol exposure in Drosophila melanogaster. Genome biology. 2006; 7:R95. [PubMed: 17054780]

Morozova TV, Anholt RR, Mackay TFC. Phenotypic and transcriptional response to selection for alcohol sensitivity in Drosophila melanogaster. Genome biology. 2007; 8:R231. [PubMed: 17973985]

Morozova TV, Ayroles JF, Jordan KW, Duncan LH, Carbone MA, Lyman RF, Stone EA, Govindaraju DR, Ellison CR, Mackay TFC, et al. Alcohol Sensitivity in Drosophila: Translational Potential of Systems Genetics. Genetics. 2009

Mulligan MK, Ponomarev I, Hitzemann RJ, Belknap JK, Tabakoff B, Harris RA, Crabbe JC, Blednov YA, Grahame NJ, Phillips TJ, et al. Toward understanding the genetics of alcohol drinking through transcriptome meta-analysis. Proceedings of the National Academy of Sciences of the United States of America. 2006; 103:6368–6373. [PubMed: 16618939]

Neuser K, Triphan T, Mronz M, Poeck B, Strauss R. Analysis of a spatial orientation memory in Drosophila. Nature. 2008; 453:1244–1247. [PubMed: 18509336]

Offenhauser N, Castelletti D, Mapelli L, Soppo BE, Regondi MC, Rossi P, D'Angelo E, Frassoni C, Amadeo A, Tocchetti A, et al. Increased ethanol resistance and consumption in Eps8 knockout mice correlates with altered actin dynamics. Cell. 2006; 127:213–226. [PubMed: 17018287]

Pandey SC, Ugale R, Zhang H, Tang L, Prakash A. Brain chromatin remodeling: a novel mechanism of alcoholism. Journal of Neuroscience. 2008; 28:3729–3737. [PubMed: 18385331]

Park SK, Sedore SA, Cronmiller C, Hirsh J. Type II cAMP-dependent protein kinase-deficient Drosophila are viable but show developmental, circadian, and drug response phenotypes. The Journal of biological chemistry. 2000; 275:20588–20596. [PubMed: 10781603]

Parr J, Large A, Wang X, Fowler SC, Ratzlaff KL, Ruden DM. The inebri-actometer: a device for measuring the locomotor activity of Drosophila exposed to ethanol vapor. Journal of Neuroscience Methods. 2001; 107:93–99. [PubMed: 11389946]

Pfeiffer BD, Jenett A, Hammonds AS, Ngo TT, Misra S, Murphy C, Scully A, Carlson JW, Wan KH, Laverty TR, et al. Tools for neuroanatomy and neurogenetics in Drosophila. Proceedings of the National Academy of Sciences of the United States of America. 2008; 105:9715–9720. [PubMed: 18621688]

Pulver SR, Pashkovski SL, Hornstein NJ, Garrity PA, Griffith LC. Temporal dynamics of neuronal activation by Channelrhodopsin-2 and TRPA1 determine behavioral output in Drosophila larvae. Journal of neurophysiology. 2009; 101:3075–3088. [PubMed: 19339465]

Reed MR. The olfactory reactions of Drosophila melanogaster Meigen to the products of fermenting banana. Physiological Zoology. 1938; 11:317–325.

Renthal W, Nestler EJ. Epigenetic mechanisms in drug addiction. Trends in molecular medicine. 2008; 14:341–350. [PubMed: 18635399]

Richmond RC, Gerking JL. Oviposition site preference in Drosophila. Behavior genetics. 1979; 9:233–241. [PubMed: 115458]

Riley BP, Kalsi G, Kuo PH, Vladimirov V, Thiselton DL, Vittum J, Wormley B, Grotewiel MS, Patterson DG, Sullivan PF, et al. Alcohol dependence is associated with the ZNF699 gene, a human locus related to Drosophila hangover, in the Irish Affected Sib Pair Study of Alcohol Dependence (IASPSAD) sample. Molecular Psychiatry. 2006; 11:1025–1031. [PubMed: 16940975]

Ritzmann RF, Tabakoff B. Dissociation of alcohol tolerance and dependence. Nature. 1976; 263:418–420. [PubMed: 987546]

Rodan AR, Kiger JA, Heberlein U. Functional dissection of neuroanatomical loci regulating ethanol sensitivity in Drosophila. Journal of Neuroscience. 2002; 22:9490–9501. [PubMed: 12417673]

Rothenfluh A, Threlkeld RJ, Bainton R, Tsai L, Lasek A, Heberlein U. Distinct Behavioral Responses to Ethanol Are Regulated by Alternate RhoGAP18B Isoforms. Cell. 2006; 127:199–211. [PubMed: 17018286]

Rubin GM, Spradling AC. Genetic transformation of Drosophila with transposable element vectors. Science (New York, NY). 1982; 218:348–353.

Scholz H, Franz M, Heberlein U. The hangover gene defines a stress pathway required for ethanol tolerance development. Nature. 2005; 436:845–847. [PubMed: 16094367]

Scholz H, Ramond J, Singh CM, Heberlein U. Functional ethanol tolerance in Drosophila. Neuron. 2000; 28:261–271. [PubMed: 11086999]

Schwenkert I, Eltrop R, Funk N, Steinert JR, Schuster CM, Scholz H. The hangover gene negatively regulates bouton addition at the Drosophila neuromuscular junction. Mechanisms of Development. 2008; 125:700–711. [PubMed: 18524547]

Singh CM, Heberlein U. Genetic control of acute ethanol-induced behaviors in Drosophila. Alcoholism: Clinical and Experimental Research. 2000; 24:1127–1136.

Sturtevant, A. A History of Genetics. Cold Spring Harbor Laboratory Press; 1967.

Szumlinski KK, Lominac KD, Oleson EB, Walker JK, Mason A, Dehoff MH, Klugmann M, Klugman M, Cagle S, Welt K, et al. Homer2 is necessary for EtOH-induced neuroplasticity. Journal of Neuroscience. 2005; 25:7054–7061. [PubMed: 16049182]

Thiele TE, Marsh DJ, Ste Marie L, Bernstein IL, Palmiter RD. Ethanol consumption and resistance are inversely related to neuropeptide Y levels. Nature. 1998; 396:366–369. [PubMed: 9845072]

Treistman SN, Martin GE. BK Channels: mediators and models for alcohol tolerance. Trends in Neurosciences. 2009; 32:629–637. [PubMed: 19781792]

Urizar NL, Yang Z, Edenberg HJ, Davis RL. Drosophila homer is required in a small set of neurons including the ellipsoid body for normal ethanol sensitivity and tolerance. Journal of Neuroscience. 2007; 27:4541–4551. [PubMed: 17460067]

Urso T, Gavaler JS, Van Thiel DH. Blood ethanol levels in sober alcohol users seen in an emergency room. Life sciences. 1981; 28:1053–1056. [PubMed: 7219065]

van Delden W, Kamping A. Genetic variation for oviposition behavior in Drosophila melanogaster. II. Oviposition preferences and differential survival. Behavior genetics. 1990; 20:661–673. [PubMed: 2126926]

Waddell S, Armstrong JD, Kitamoto T, Kaiser K, Quinn WG. The amnesiac gene product is expressed in two neurons in the Drosophila brain that are critical for memory. Cell. 2000; 103:805–813. [PubMed: 11114336]

Wang Y, Ghezzi A, Yin JC, Atkinson NS. CREB regulation of BK channel gene expression underlies rapid drug tolerance. Genes, Brain and Behavior. 2009; 8:369–376.

Wang Y, Krishnan HR, Ghezzi A, Yin JC, Atkinson NS. Drug-induced epigenetic changes produce drug tolerance. PLoS biology. 2007; 5:e265. [PubMed: 17941717]

Watabe M, Aoyama K, Nakaki T. Regulation of glutathione synthesis via interaction between glutamate transport-associated protein 3–18 (GTRAP3-18) and excitatory amino acid carrier-1 (EAAC1) at plasma membrane. Molecular pharmacology. 2007; 72:1103–1110. [PubMed: 17646425]

Wen T, Parrish CA, Xu D, Wu Q, Shen P. Drosophila neuropeptide F and its receptor, NPFR1, define a signaling pathway that acutely modulates alcohol sensitivity. Proceedings of the National Academy of Sciences of the United States of America. 2005; 102:2141–2146. [PubMed: 15677721]

Wolf FW, Rodan AR, Tsai LT, Heberlein U. High-resolution analysis of ethanol-induced locomotor stimulation in Drosophila. Journal of Neuroscience. 2002; 22:11035–11044. [PubMed: 12486199]

Table 1

Behavioral ethanol phenotypes of selected *Drosophila* mutations.
The table includes mutant alleles that have been studies in some detail, and where there is some understanding on the molecular effects of the mutant.

Gene	Encoded Protein	Pathway	Phenotype	Reference
		signaling		
GABA(B)	metabotropic receptor	GABA	lof: **AR**	Dzitoyeva et al., 2003
TH	tyrosin hydroxylase	dopamine	lof: **AYD**	Bainton et al., 2000
DopR	dopamine D1 receptor	dopamine	lof: **AYD**	Kong et al., 2010b
Tbh	tyramine β-hydroxylase	octopamine	lof: A+, **RTD**	Scholz et al., 2000
InR	insulin receptor	insulin	lof: **AS**	Corl et al., 2004
chico	insulin receptor substrate	insulin	lof: **AS**	Corl et al., 2004
FOXO	transcription factor	insulin	gof: **AS**	Corl et al., 2004
NPF	neuropeptide F (NPY)	NPY	gof: **AS**	Wen et al., 2005
cheapdate	amnesiac/PACAP	cyclic AMP	lof: **AS**	Moore et al., 1998
rutabaga	adenylate cyclase	cyclic AMP	lof: **AS**	Moore et al., 1998
DCO	protein kinase A	cyclic AMP	lof: **AS**	Moore et al., 1998, Rodan et al., 2002
DCO	protein kinase A	cyclic AMP	lof: **AR***(1)*	Rodan et al., 2002
pka-RII	PKA regulation	cyclic AMP	lof: **AS**	Park et al., 2000
happy hour	Ste20 kinase	EGF	lof: **AR**, gof: **AS**	Corl et al., 2009
Egfr	EGF receptor	EGF	lof: **AS**, gof: **AR**	Corl et al., 2009
Spitz	EGF ligand	EGF	gof: **AR**	Corl et al., 2009
rhomboid	ligand-activating peptidase	EGF	lof: **AS**	Corl et al., 2009
rolled	ERK kinase	EGF	gof: **AR**	Corl et al., 2009
elm	P22 calcineurin B homolog	Ca-binding	lof: **AS**	LaFerriere et al., 2008
		synapse function		
slowpoke	BK channel	membrane potential	lof: **RTD**, gof: **AR**	Cowmeadow et al., 2005, 2006
synapsin	presynaptic scaffolding	synaptic plasticity	lof: A+, **RTI**	Godenschwege et at., 2004
homer	postsynaptic scaffolding	synaptic plasticity	lof: **AS**, **RTD**	Urizar et al., 2007
		cell structure		
fasII	fasciclinII/NCAM2	cell adhesion	lof: **AS**	Cheng et al., 2001
myospheroid	β-integrin	cell adhesion	lof: **AS**, **RTI**	Bhandari et al., 2009

Gene	Encoded Protein	Pathway	Phenotype	Reference
scabrous	a-integrin	cell adhesion	lof: **AS**, **RTI**	Bhandari et al., 2009
white rabbit-RA	RhoGAP18B-PA*(2)*	actin dynamics	lof: **AYD**, gof: **AYI**	Rothenfluh et al., 2006
white rabbit-RC	RhoGAP18B-PC*(2)*	actin dynamics	lof: **AR**	Rothenfluh et al., 2006
Rho1	RhoGTPase	actin dynamics	gof: **AR**	Rothenfluh et al., 2006
Rac1	RhoGTPase	actin dynamics	lof: **AS**, gof: **AR**	Rothenfluh et al., 2006
Cdc42	RhoGTPase	actin dynamics	lof: **AR**, gof: **AS**	Rothenfluh et al., 2006
jwa	ARL6IP5	microtubule assoc.	lof: **RTD**, gof: **RTI**	Li et al., 2008
		DNA regulation		
Sir2	histone deacetylase	chromatin regulation	lof: **AR**, **RTD**	Kong et al., 2010
pipsqueak	helix-turn-helix protein	transcription factor	gof: **AS**	Morozova et al., 2009
hangover	Zinc-finger protein	transcription factor	lof: A+, **RTD**	Scholz et al., 2005
		metabolism		
Men	malic enzyme	intermediate metab.	lof: **AR**	Morozova et al., 2009
AcCoAS	acetyl CoA synthase	intermediate metab.	lof: A+, **AYD**	Kong et al., 2010a

Abbreviations: loss-of-function (lof), gain-of-function (gof), acute sedation sensitivity (AS), acute sedation resistance (AR), no change in acute sedation (A+), decreased acute hyperactivation (AYD), increased acute hyperactivation (AYI), decreased rapid tolerance (RTD), increased rapid tolerance (RTI).

*(1)*Ubiquitous inhibition of PKA causes acute sensitivity, while inhibiting in a subset of neurons only can also lead to acute resistance.

*(2)*Two different isoforms from the same gene, sharing the GTPase activating domain, but with different N-termini.