

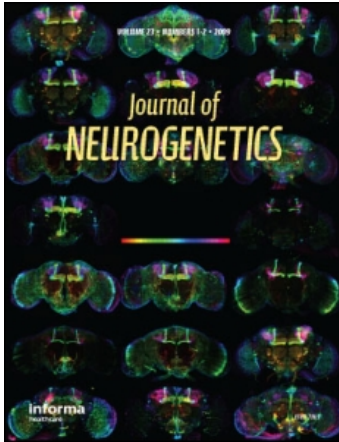
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Journal of Neurogenetics

Publication details, including instructions for authors and subscription information:

<http://www.informaworld.com/smpp/title-content=t713644816>

Tolerance in *Drosophila*

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First Published on: 29 January 2009

To cite this Article Atkinson, Nigel S.(2009)'Tolerance in *Drosophila*',*Journal of Neurogenetics*,23:3,293 — 302

To link to this Article: DOI: 10.1080/01677060802572937

URL: <http://dx.doi.org/10.1080/01677060802572937>

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Tolerance in *Drosophila*

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Abstract: The set of genes that underlie ethanol tolerance (inducible resistance) are likely to overlap with the set of genes responsible for ethanol addiction. Whereas addiction is difficult to recognize in simple model systems, behavioral tolerance is readily identifiable and can be induced in large populations of animals. Thus, tolerance lends itself to analysis in model systems with powerful genetics. *Drosophila melanogaster* has been used by a variety of laboratories for the identification of genes that interfere with the acquisition of ethanol tolerance. Here, I discuss the genes identified as being important for the production of ethanol tolerance in *Drosophila*. Some of these genes have also been shown to be important for the production of tolerance in mammals, demonstrating that gene discovery in *Drosophila* has predictive value for understanding the molecular pathways that generate tolerance in mammals.

Keywords: *Drosophila*, alcohol, tolerance, addiction, gene, behavior, fly

The acquisition of drug tolerance is an important component of the addictive process. Tolerance is defined as a reduction in an effect of a drug caused by prior drug exposure. In humans, tolerance can be an insidious response to an abused drug, leading to increased consumption and speeding the user down the path to addiction. Although the details are not yet known, it is clear that addiction is caused by drug-induced changes in neural activity that cause complex behavioral changes. These changes result in the pursuit and consumption of the drug to the detriment of the individual, often despite obvious drug-induced illness and financial and personal loss. The molecular underpinnings of tolerance and addiction are likely to be intimately related. Thus, understanding the molecular origins of tolerance is important for our understanding of the addictive process (Nestler, 2005).

The lexicon of this field is rich in words that have subtle, but very important, distinctions in meaning. Tolerance is different from drug resistance. The term resistance refers to a comparison of the drug sensitivity of two animals or genotypes and indicates that one requires a larger dose of the drug to experience the same effect, whereas tolerance refers to a change in sensitivity to the drug from one exposure to another. The key distinction is that resistance occurs in drug-naïve animals, whereas tolerance (i.e., inducible resistance) requires prior drug exposure. One should also be aware that, in the

Drosophila ecology and evolution field, the term “ethanol tolerance” does not mean inducible resistance, but has been used to mean innate resistance to the toxic effects of ethanol. This latter meaning is not used in pharmacology.

There are a number of distinct types of drug tolerance. Conceptually, tolerance is divided into categories based on the method of induction. Acute tolerance occurs during the drug experience, whereas rapid tolerance arises after a single drug exposure—after the drug has been cleared (usually within 24 hours). Chronic tolerance appears following multiple or prolonged drug exposure. It is likely that these forms of tolerance have related, but not completely overlapping, mechanisms (Kalant, 1996; Berger et al., 2004).

Tolerance is also subdivided, based on mechanistic differences. Metabolic or pharmacokinetic tolerance reflects an increase in the rate of catabolism of the drug caused by prior drug exposure. In mammals, metabolic (i.e., pharmacokinetic) tolerance often involves the induction of liver enzymes that act to clear the drug (Julien, 2004).

More interesting to a neurobiologist is tolerance that arises because of metabolism-independent changes that cause the nervous system to be less responsive to the drug. This type of tolerance is referred to as pharmacodynamic tolerance, or more simply, functional tolerance. Functional tolerance is thought to involve plastic changes

Received 22 July 2008; Accepted 17 October 2008

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in the nervous system that are similar to those involved in learning and memory. Further, many of the same genes involved in learning and memory appear to contribute to functional tolerance (Berger et al., 2008).

A robust literature points to the influence of genetic factors in drug addiction and in alcoholism (reviewed in Kendler et al. 2003; Mayfield et al. 2008). However, the identification of the responsible genes in mammals is difficult because generating mutants in mammals is time-consuming and expensive. *Drosophila melanogaster* provides an attractive alternative to mammalian systems for the high-throughput mutant analysis of genes and is the preeminent model animal for the genetic manipulation of genes. The *Drosophila* community maintains a large, freely available collection of mutant flies in its stock centers. At this time, this collection includes mutants in ~70% of *Drosophila* genes (Venken & Bellen, 2005). It is predicted that within a few years, the available collections will contain mutations for essentially every gene. Thus, in *Drosophila*, it is possible to use mutant analysis to test genes more rapidly than can be done with mammals.

With any model system, there is a trade-off between simplicity and applicability, but *Drosophila* offers an attractive compromise. Despite having a much smaller genome (with approximately 180 million base pairs to a human's 3.2 billion), there is a surprising degree of gene homology (Adams et al., 2000; Makalowski, 2001). Further, *Drosophila* and humans have a similar number of genes (Celniker & Rubin, 2003; Abdellah et al., 2004). Most or all genes found in mammals are represented in *Drosophila* and most of the important developmental regulatory cascades were discovered in *Drosophila*. Of 929 human disease genes, 714 had counterparts among 548 distinct *Drosophila* genes (Reiter et al., 2001).

Drosophila exhibit many of the same behaviors as humans. They sleep, learn, remember, court, and fight (Quinn et al., 1974; Hall, 1994; Hendricks et al., 2000; Chen et al., 2002). Further, *Drosophila* and humans have similar pharmacological and behavioral responses to a number of abused drugs (reviewed in Hirsh, 2001; Wolf & Heberlein, 2003; Nichols, 2006; Heberlein et al., 2008). Most important for this review, *Drosophila* show a pattern of ethanol intoxication that parallels that of humans, with an initial phase of excitation followed by sedation (Bainton et al., 2000; Singh & Heberlein, 2000). In both flies and mammals, low doses of ethanol are excitatory and higher doses produce incoordination and sedation. *Drosophila* also manifest rapid, chronic functional tolerance to the sedative effects of ethanol (Scholz et al., 2000; Berger et al., 2004; Cowmeadow et al., 2005).

At a molecular level, *Drosophila* and mammalian neurons are very clearly homologous (discussed in detail in Wolf & Heberlein, 2003). For this reason, *Drosophila*

genes are often useful as tools to identify and clone mammalian homologs. However, because the circuitry of the *Drosophila* brain is substantially different than the mammalian brain, and because behavior is a systemic, emergent response of the nervous system, it is reasonable to expect that even drugs that have the same cellular target may not have the exact same behavioral consequence. *Drosophila* mutations that perturb drug responses may not produce the same phenotype as the corresponding mammalian mutation. A mutant-induced change in drug responsiveness merely indicates that the gene is important in response to the drug, not how the mutation will affect drug responsiveness in mammals. The mutants discussed in this review are summarized in Table 1.

This review will focus on recent work on ethanol tolerance, using the model organism, *Drosophila melanogaster*. For the fly geneticist, alcohol tolerance is an attractive phenotype for study because it is a satisfying compromise between complexity and simplicity. Tolerance reduces the effect of the drug on the animal, suggesting that it is a homeostatic response whose purpose is to restore the status quo. Adult flies are a particularly interesting subject for this study because they do not acquire metabolic tolerance to ethanol (a.k.a. pharmacokinetic tolerance). That is, adult flies do not change the rate of ethanol metabolism in response to prior ethanol exposure (Scholz et al., 2000; Berger et al., 2004; Cowmeadow et al., 2005). This may be because adult ADH activity is not induced by ethanol exposure (Geer et al., 1988). In any case, the lack of metabolic tolerance makes adult flies an excellent system for the study of functional (i.e., pharmacodynamic) tolerance. Therefore, in flies, tolerance to the sedating effects of alcohol must arise because of changes in the responsiveness of the nervous system. The consideration of tolerance as a form of neural plasticity has led investigators to ask whether the manifestation of functional tolerance is mediated by the same genes that mediate other forms of neural plasticity, such as learning and memory.

Tolerance is far simpler to study than addiction. In humans, addiction appears to have a strong genetic component whose penetrance is influenced by experience. Unfortunately, there is no simple behavioral assay for addiction, alcoholism, or dependence (DSM-IV, 1994). Functional tolerance, on the other hand, can be reliably induced in populations of animals, and can be easily scored by using simple behavioral assays. Because tolerance can be induced and assayed in populations of animals, it is ideal for study in high-throughput genetic systems such as *Drosophila melanogaster*.

A small number of behavioral assays have been used to identify mutations that interfere with the ability of flies to acquire tolerance. Multiple laboratories have made excellent use of the *Inebriometer* (see below), a device consisting of a large vertical tube which contains a series

Table 1. Genes affecting the acquisition of ethanol tolerance in *Drosophila*

Category	Gene(s)	Relevant phenotype	Reference
neurotransmitter biosynthesis	<i>tyramine β-hydroxylase</i>	mutant has a 50–60% reduction in the magnitude of ethanol tolerance	Scholz et al. 2000
cellular stress	<i>hangover</i> (Zn finger containing protein)	mutant has a ~60% reduction in the magnitude of ethanol tolerance	Scholz et al. 2005 Riley et al. 2006
	<i>djwa</i>	RNAi blocks tolerance in knock-down and duration of sedation assays	Li et al. 2008
memory	14 mutant animals	reduction in ability to acquire rapid &/or chronic tolerance, with the exception of the john mutant which shows increased chronic tolerance.	Berger et al. 2008
synaptic transmission modulation of neuronal excitability	<i>synapsin</i> <i>GABA_B-R1 & -R2</i> (metabotropic GABA receptors)	mutant has enhanced rapid ethanol tolerance pharmacological inhibition prevents ethanol tolerance	Godenschwege et al. 2004. Dzitoyeva et al. 2003 Mezler et al. 2001
synaptic structure	<i>homer</i>	increased ethanol sensitivity, decreased ethanol tolerance	Urizar et al. (2007) Diagana et al. (2002) Szumlinski et al. (2005)
olfaction & biotransformation pathways	A variety of genes	some mutants affect tolerance without affecting ethanol sensitivity	Morozova et al. (2006)
potassium channel	<i>slo</i> (BK type Ca ²⁺ -activated K ⁺ channels)	inability to acquire rapid tolerance in a duration of sedation assay	Ghezzi et al. 2004, Cowmeadow et al. 2005 & 2006, Davis et al. 2003, Pietrzykowski et al. 2004

of baffles. Flies are added at the top of the tube and ethanol vapor is passed through the tube. Flies are normally negatively geotactic and cling to the baffles in order to remain at the top of the tube. As the flies succumb to the effects of the ethanol, they lose the ability to hang on to the baffles and so fall through the tube (Cohan & Graf, 1985). The amount of time that the flies remain in the column is a measure of their ethanol sensitivity.

Thus, this device can be used to sort populations for animals that behave differently (either eluting more rapidly or later than their contemporaries), and the mean elution time of a population serves as a numerical measure of the response of a population to ethanol. Tolerance can be quantified based on the difference between the mean elution time of naïve animals and the mean elution time of animals that have been previously exposed to ethanol. Flies that have acquired ethanol tolerance elute from the column later than their naïve counterparts (Scholz et al., 2000).

A large collection of mutations that perturb alcohol tolerance have been collected with this device. Members of the Heberlein laboratory popularized the use of the *Inebriometer* for the identification of mutations altering the capacity to acquire ethanol tolerance. Some of these mutations alter genes whose role can be rationalized based on current knowledge, but some mutations clearly

identify novel targets whose role in alcohol responsiveness was not anticipated. Substantial evidence indicates that many of the genes identified in *Drosophila* will be useful for understanding ethanol tolerance in mammals.

TYRAMINE β-HYDROXYLASE

Scholz et al. (2000) showed that a mutation in *Tbh*, the gene encoding tyramine β-hydroxylase, which interferes with octopamine synthesis, also impairs the ability of flies to acquire tolerance. In insects, octopamine is proposed to subserve many of the same functions as noradrenaline in mammals (Monastirioti et al., 1996). Interestingly, noradrenaline has previously been implicated in the production of functional tolerance in mammals (Ritzmann & Tabakoff, 1976). Mutations such as these help to identify parallels between invertebrate and vertebrate systems.

CELLULAR STRESS-RESPONSE GENES

In a subsequent study, Scholz et al. (2005) went on to use the *Inebriometer* in an unbiased genetic screen and identified an intriguing mutation in a gene that they named *hangover*. A null mutation in *hangover* (*hang*^{AE10}) reduces the magnitude of tolerance induced by a single

ethanol exposure (rapid tolerance) without altering the flies' initial sensitivity to ethanol. The *hangover* gene encodes 15 Zn-fingers motifs (two of which share a similarity to U1 RNA binding proteins) and an EF-hand motif, suggesting that the gene participates in RNA processing, localization, or stability and is responsive to Ca^{2+} (Scholz et al., 2005).

Because the *hang*^{AE10} allele causes flies to be more sensitive to paraquat-induced oxidative stress, the *hangover* gene was also postulated to be involved in responses to cellular stressors. In this context, ethanol is viewed as one of many possible stressors (Scholz et al., 2005).

Additionally, the Hangover protein is a negative regulator of the growth of synaptic boutons. Larvae mutants for *hangover* have an increased number of boutons at the larval neuromuscular junction, although overall synaptic transmission appears normal. Hangover is thought to mediate this phenotype indirectly by affecting the expression of other genes. One likely regulatory target of *hangover* is the *fasciclin II* (*fasII*) gene product. Expression of the FASII cell-adhesion molecule is reduced in *hangover* mutants (Schwenkert et al., 2008). A reduction in *fasII* expression has been previously shown to cause increased proliferation at the neuromuscular junction (NMJ) without a parallel increase in synaptic strength (Schuster et al., 1996). However, unlike mutations in *hangover*, mutations in *fasII* cause increased ethanol sensitivity in naïve animals (Cheng et al., 2001). Nevertheless, *hangover* mutations may affect the capacity for ethanol tolerance via an effect on *fasII* expression.

The role of the mammalian *hangover* homolog in ethanol responses has not yet been extensively studied. However, a large sibling pair study of alcohol dependence in humans showed genetic linkage between clinically defined alcohol dependence and seven single-nucleotide polymorphisms (SNPs) linked to a human *hangover* homolog (Riley et al., 2006).

Another gene linked to the response to cellular stressors is JWA. Homologs to JWA are referred to by multiple names in the literature (*addiccin*, *GTRAP3-18*, and *Arl6ip5*; Zhu et al., 2006). The protein is thought to be involved in the regulation of glutamate/aspartate transport in the central nervous system (CNS) (Lin et al., 2001) and in cellular responses to environmental stressors (based on the observation that expression is induced by cellular stressors; Zhu et al., 2006). The *Drosophila* homolog is referred to as *djwa*. Li et al. (2008) demonstrated that a reduction of *djwa* mRNA abundance by RNAi prevents adult flies from acquiring tolerance, as assayed by either an inebriometer knock-down assay or a duration of a sedation assay. As of yet, there has been no test to determine if *hangover* and *djwa* act in the same or in parallel pathways.

MEMORY MUTANTS

It has long been suspected that the brain “learns” to be addicted, and that the same genes important for learning and memory would participate in the addictive process (reviewed in Hyman et al. (2006)).

Many of the original *Drosophila* learning and memory mutants perturbing cAMP signaling were shown to affect alcohol sensitivity (Moore et al., 1998). Recently, in Berger et al. (2008), the Heberlein laboratory revisited this question of whether mutations affecting learning and memory also affect alcohol responses. They made use of the *Inebriometer* to examine a large collection of *Drosophila* mutants that were defective in long-term memory (Dubnau et al., 2003).

Mutant alleles of 52 distinct genes, identified as causing a defect in long-term memory, were examined for their capacity to acquire rapid and chronic tolerance (Berger et al., 2008). Rapid tolerance was measured by splitting a matched population into two groups and treating one group with a 60/40 mix of ethanol-saturated air and humidified air, while the other group (control) experienced an equivalent ethanol-free airstream. Three and one half hours later, both groups were tested in the *Inebriometer* in a 55/45 mix of ethanol vapor and humidified air. To assay chronic tolerance, the flies were exposed to a nonintoxicating dose of ethanol for 18–24 hours and then tested in the *Inebriometer*. All of these are P-element mutants (transposon insertions). In FlyBase (Grumblin et al., 2006), some of these P-elements are identified as being insertional mutants in specific genes (*exba*^{krasavietz}, *klg*^{ruslan}, *pxb*^{baik}, *rho*^{iks}, *pum*^{milord-1}). However, reversion studies have not yet been completed to confirm that the mutant phenotype segregates with the transposon insertion.

Of the original 52 genes, this screen identified 14 P-element mutants that alter the capacity to acquire rapid or chronic tolerance. Five of the P elements are positioned between genes, and the mutant phenotype has not yet been definitively mapped to individual genes. These genes are likely to be novel genes. The remainder are thought to be transposon insertions affecting genes not traditionally suspected as being associated with drug responses. Three of the transposon insertions are in genes that have been previously shown to affect neurogenesis or axon guidance (*exba*^{krasavietz}, *klg*^{ruslan}, *pxb*^{baik}), while two are known to be involved early and often in embryonic development (*rho*^{iks}, *pum*^{milord-1}) (Grumblin et al., 2006).

Two of the mutations affecting tolerance that seem most interesting are *exba*^{krasavietz} and *klg*^{ruslan}, which reduce the capacity to acquire both rapid and chronic tolerance. It is likely that rapid and chronic tolerance will

have some overlapping mechanisms and that these mutations perturb a common component.

Only one mutant showed an increase in the magnitude of tolerance. This mutant line, referred to as “john,” showed increased chronic tolerance, but no obvious alteration in the capacity for rapid tolerance. However, for these animals, chronic ethanol exposure causes a reduction in ethanol content in response to a test exposure. Presumably, the mutation causes an inducible enhancement in ethanol clearance, that is, metabolic (pharmacokinetic) tolerance.

SYNAPSIN

Synapsins are neurotransmitter vesicle-associated proteins that are important for normal synaptogenesis and for neural plasticity (Greengard & Browning, 1988). Synapsins appear to be involved in the segregation of vesicles between the reserve and releasable pools (Llinas et al., 1985). In mice, mutant alleles of synapsins I, II, or III affect synaptic plasticity (Rosahl et al., 1993, 1995; Feng et al., 2002). However, it is difficult to determine whether the loss of all Synapsin expression would have more profound consequences. In *Drosophila*, the synapsin gene family is represented by a single member. The reduction in the number of gene family members is a common occurrence in flies and enables one to more easily determine the consequence of eliminating all examples of a particular class of protein.

Godenschwege et al. (2004) generated a null mutation in the *Drosophila* synapsin gene. The mutant animals are healthy and fecund, have an ultrastructurally normal nervous system, and show no gross differences in behavior when compared to wild type. However, detailed and extensive behavioral studies reveal that the synapsin null mutants have a learning and/or memory deficit and perform poorly in a variety of learning and memory tests.

A simplified rapid tolerance assay was used to determine if the mutation altered the response to alcohol. Rapid tolerance was induced by exposing flies to 50% ethanol vapor until half of the animals were motionless. Four hours later, the assay was repeated to quantify tolerance (defined as an increase in the time required to sedate half of the population). The synapsin null mutation enhanced the magnitude of acquired tolerance. This is noteworthy, because it is rather uncommon for a mutation to increase the capacity to acquire tolerance. These results suggest that the mechanism by which the reserve pool of synaptic vesicles is maintained is an integral part of the production of tolerance (Hilfiker et al., 1999; Godenschwege et al., 2004).

GABA_B RECEPTORS

The Manev laboratory has taken an innovative, novel approach to studying tolerance in *Drosophila*. This laboratory pioneered the use of direct intra-abdominal injections of adult flies as a means to deliver ethanol, drugs, and even dsRNAi triggers into the nervous system of the fly (Dzitoyeva et al., 2001, 2003). In their approach, carbon dioxide (CO₂)-anesthetized flies are injected with ethanol to induce sedation. The control flies are injected with vehicle, and the period of ethanol-induced sedation is measured (flies recover almost immediately from CO₂ anesthesia). To test for the capacity to acquire tolerance, two consecutive injections are delivered (800 nmol of ethanol per fly). The first ethanol injection induces tolerance that persists for about 18 hours.

This group examined the role of the recently discovered *Drosophila* GABA_B receptors (Mezler et al., 2001) in the production of ethanol tolerance in *Drosophila*. In mammals, GABA_B receptor agonists interrupt the acquisition of rapid tolerance to ethanol intoxication (Zaleski et al., 2001). Dzitoyeva et al. (2003) demonstrated that direct injections of the GABA_B agonist (3-APMPA) also blocked rapid ethanol tolerance in flies. These investigators also showed that the ethanol motor impairment of flies was partially alleviated by injecting adults with dsRNAi triggers specific for the GABA_BR1 receptor as a means to reduce GABA_BR1 mRNA abundance. This work further illustrates the strong molecular similarity of ethanol targets in mammals and flies.

A very important aspect of this work and subsequent publications on this topic is the demonstration of the ease with which individual adult flies can be injected with drugs and pharmaceuticals (Manev et al., 2003). Such manipulations were previously not considered possible in *Drosophila* and their absence had been a substantial limitation with regard to *Drosophila* pharmacology. Further, the injection of adults as a means of triggering RNAi-mediated destruction of mRNAs is also significant. Mutant phenotypes can be tested in the absence of any developmental defect. Even for *Drosophila*, the generation of mutants and transgenic animals can be more time-consuming than the RNAi-mediated manipulation of gene expression in adults.

homer

Urizar et al. (2007) employed a novel tolerance protocol in which flies were exposed to a large dose of ethanol vapor (50–70% ethanol), allowed to recover in the presence of 10% ethanol vapor, and then re-exposed to 50–70% ethanol vapor. Individual flies were visually scored to determine when they were sedated. During the

second exposure, fewer flies were sedated at the end of a 40-minute test period, indicating that tolerance was induced. The investigators described this as a rapid tolerance protocol. However, rapid tolerance has conventionally been defined as tolerance induced by a single short exposure that can be measured after the ethanol is completely metabolized (Berger et al., 2004). In this particular paradigm, the flies are never fully deprived of ethanol, and therefore, the drug cannot be completely cleared prior to the second exposure. Therefore, this protocol might better be classified as a chronic tolerance protocol. This is a small point, but it is worth noting, should rapid and chronic tolerance eventually be shown to be mechanistically distinct. The chronic tolerance protocol of Urizar et al. (2007) was similar to their rapid tolerance protocol, except that the flies received five consecutive exposures to concentrated ethanol vapor, followed by 10% ethanol vapor for 24 hours.

Both treatment protocols caused a slight reduction in the relative abundance of transcripts from the *homer* gene. In mammals, *homer* genes encode postsynaptic proteins that regulate or maintain glutamatergic synapses, and *homer* genes have been linked to drug-induced neuroplasticity (recently reviewed by Szumlinski et al., 2008). Ethanol induces mouse *homer2* expression, and *homer2* knockout mice show altered ethanol responses, including alcohol avoidance, increased alcohol-induced sedation, and a failure to acquire tolerance in a locomotor assay (Szumlinski et al., 2005).

In flies, a *homer* mutant (*homer*^{R102}) was first described as being hyperactive and defective in associative learning (Diagana et al., 2002). Urizar et al. (2007) demonstrated that the *homer*^{R102} mutation also increases the sensitivity of flies to ethanol sedation and reduces the capacity to acquire ethanol tolerance. This mutant phenotype could be complemented by transgenic expression of the wild-type Homer protein in the nervous system.

MICROARRAYS AND THE INEBRIOMETER

Morozova et al. (2006) used microarray analysis to identify genes whose expression changes concomitant with the appearance of tolerance. Tolerance was induced by passing flies through an *Inebriometer* filled with undiluted ethanol vapor. As all such microarray assays have shown to date, these assays showed that the ethanol exposure changed the abundance of mRNAs from a large number of genes. However, none of the genes discussed above showed changed expression levels. Possible explanations for this are that the mRNAs of these other genes are of extremely low abundance, that ethanol induces such a small change in their expression as to be statistically invisible in their microarray analysis, that

differences in the tolerance induction protocol produce significant differences in gene expression, or that only post-transcriptional regulation of these gene products participates in the production of tolerance.

Among the genes that change in response to the induction of tolerance, 20 were chosen for mutant analysis. The selection process did not involve preconceptions concerning the mechanics of ethanol responsiveness; rather, the genes were chosen based solely on whether a P-element (transposon) mutant of them existed in an isogenic background.

Some of these mutations affected only sensitivity and some affected only tolerance. Some mutations, which did not affect initial ethanol sensitivity, nevertheless enhanced the magnitude of tolerance, while others reduced the magnitude of tolerance. This observation leads to the surprising conclusion that development of tolerance is, at least partially, independent of initial ethanol sensitivity.

The genes identified as influencing tolerance were not the "usual suspects," but instead featured genes associated with pyruvate metabolism and fatty-acid synthesis biotransformation pathways, transcriptional regulation, and proteolysis. Analysis of such mutants is likely to broaden our understanding of the mechanisms underlying functional tolerance.

BK-TYPE Ca^{2+} -ACTIVATED K^{+} CHANNELS

My laboratory studies the role of BK-type Ca^{2+} -activated K^{+} channels in the production of rapid ethanol tolerance. The pore-forming subunits of these channels are encoded by a single gene in both mammals and invertebrates. This gene, *slo* (a.k.a. *slowpoke*, *slo-1*, *hsls*, or *mslo*, depending on the species), is highly conserved in both sequence and function (Atkinson et al., 1991; Schreiber & Salkoff, 1997; Schreiber et al., 1999; Piskorowski & Aldrich, 2002; Davies et al., 2003). The *slo*-encoded BK channels have the highest conductance of any described K^{+} channels (depending on cell type, at least 10 to 20 times as great as other K^{+} channels), and their gating mechanism integrates calcium, electrical, and metabolic signals. These channels are widely expressed in the nervous system and play a central role regulating neural activity (Rudy, 1988; Gribkoff et al., 2001). In the last few years, a strong case has emerged implicating *slo* channels as an important ethanol target and as a modulator of behavioral responses to ethanol.

The activity of *slo* channels has been shown to be potentiated by ethanol in both mammals and in *Caenorhabditis elegans*, indicating that this channel is an evolutionarily conserved ethanol target (Dopico et al., 1999; Davies et al., 2003; Liu et al., 2003; Pietrzykowski et al., 2004; Crowley et al., 2005). Further, an unbiased

C. elegans screen for mutations that cause alcohol resistance identified multiple mutant alleles of the *slo* gene (Davies et al., 2003).

In mammals, *slo* channels have been implicated in the development of functional tolerance. For example, in rat neuroendocrine cells, ethanol directly potentiates BK channel activity and is thought to contribute to ethanol suppression of neuropeptide secretion (Dopico et al., 1996; Knott et al., 2002). In these cells, alcohol tolerance mechanistically arises from 1) phosphorylation of extant BK channels, which reduces ethanol potentiation of the channels; 2) the internalization of pre-existing BK channels; and 3) a change in *slo* mRNA splicing to favor the production of splice variants that encode replacement channels that are ethanol resistant (Roberto et al., 2006).

Working with *Drosophila*, we obtained independent evidence for a role of the *slo* gene in ethanol tolerance. We measure tolerance, not as a reduced rate of sedation, but as a decrease in the duration of sedation (as determined by visual inspection). This form of tolerance is apparent 4 hours after the first sedation and persists for about 7 days. A mutant combination that eliminates *slo* expression only in the nervous system completely eliminates the ability to acquire tolerance in this assay (Cowmeadow et al., 2005).

The *Drosophila slo* gene has a complex promoter region with five tissue-specific promoters (Bohm et al., 2000). Sedation with ethanol induces expression specifically from the two neural promoters. Further, we have shown that increasing expression of a *slo* mRNA neural splice variant from a transgene phenocopies the tolerance phenotype. Thus, in flies, the *slo* gene appears to play a pivotal role in the acquisition of rapid tolerance (Cowmeadow et al., 2006).

In related work, we characterized the role of *slo* in rapid tolerance to the anesthetic benzyl alcohol (Ghezzi et al., 2004). For this drug, we have described the transcriptional mechanism underlying sedation-induced *slo* expression. At least for benzyl alcohol, induction is mediated by the CREB transcription factor (Wang et al., 2007). At this time, it is only an assumption that ethanol induction of *slo* expression depends on the same transcriptional mechanism.

Our work leads one to the counterintuitive conclusion that the increased *slo* channel expression acts as a neural excitant that counters the sedating effects of drug sedation. This is counter to the dogmatic view that increased K⁺ channel activity is always correlated with the depression of neural activity. However, the literature is rich with examples of increased Ca²⁺-activated K⁺ channel activity *reducing* the neural refractory period while *enhancing* the firing rate of neurons (Warbington et al., 1996; Lovell & McCobb, 2001; Pattillo et al., 2001; Van Goor et al., 2001; Brenner et al., 2005).

It is highly significant that the same ion-channel gene has been independently shown to be involved in ethanol responses in nematodes, insects, and mammals. This may mean that ethanol targets a highly conserved region of the channel that is required for normal channel activity. Alternatively, the conserved attribute may not be within the sequence of the channel itself, but in a conserved function subserved by the channel in neurons.

CONCLUSION

Although the study of ethanol tolerance in flies has attracted only a small number of fly labs, a large number of parallels between the molecular biology of the response in insects and mammals has been uncovered. The *Drosophila* model system, with its powerful genetics, has much to offer to the understanding of how addictive drugs induce functional tolerance.

ACKNOWLEDGEMENTS

The author would like to thank the members of his lab who made suggestions that improved this manuscript and would especially like to thank Jane Kirschman, Roseanna Ramazani, Jascha Pohl, and Harish Krishnan for being responsive each time the author asked for readers.

NSA is supported by the National Institutes of Health (grant no. RO1 DA022219) and the National Science Foundation (grant no. IOS-0641370).

Declaration of interest: The author reports no financial conflicts of interest. The author alone is responsible for the content and writing of this paper.

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This paper was first published online on iFirst on 29 January 2009.