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Large analysis of genetic manipulations reveals an inverse correlation between initial alcohol resistance and rapid tolerance phenotypes

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Abstract

Tolerance occurs when, following an initial experience with a substance, more of the substance is required subsequently to induce identical behavioral effects. Tolerance is not well-understood, and numerous researchers have turned to model organisms, particularly *Drosophila melanogaster*, to unravel its mechanisms. Flies have high translational relevance for human alcohol responses, and there is substantial overlap in disease-causing genes between flies and humans, including those associated with Alcohol Use Disorder. Numerous *Drosophila* tolerance mutants have been described; however, approaches used to identify and characterize these mutants have varied across time and labs and have mostly disregarded any impact of initial resistance/sensitivity to ethanol on subsequent tolerance development. Here, we analyzed our own, as well as data published by other labs to uncover an inverse correlation between initial ethanol resistance and tolerance phenotypes. This inverse correlation suggests that initial resistance phenotypes can explain many 'perceived' tolerance phenotypes, thus classifying such mutants as 'secondary' tolerance mutants. Additionally, we show that tolerance should be measured as a relative increase in time to sedation between an initial and second exposure rather than an absolute change in time to sedation. Finally, based on our analysis, we provide a method for using a linear regression equation to assess the residuals of potential tolerance mutants. These residuals provide predictive insight into the likelihood of a mutant being a 'primary' tolerance mutant, where a tolerance phenotype is not solely a consequence of initial resistance, and we offer a framework for understanding the relationship between initial resistance and tolerance.

Abbreviations: AUD, alcohol use disorder; CNS, central nervous system; GOF, gain-of-function; HDM, histone demethylase; LOF, loss-of-function; LORR, loss of righting reflex; ST-50, time to 50% sedation.

Maggie M. Chvilicek and Alexandra Seguin contributed equally.

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KEYWORDS
Drosophila, ethanol, genetics, sensitivity, tolerance

1 | INTRODUCTION

Alcohol Use Disorder (AUD) is a major public health and societal problem. According to the 2021 National Survey on Drug Use and Health, 60 million people aged 12 years or older were binge drinkers in the past month, and 29.5 million people 12 years and older reported having a diagnosed AUD in the past year. Two risk factors for AUD are resistance to the initial intoxicating effects of alcohol and elevated alcohol tolerance. Tolerance to ethanol occurs when, after an initial experience with the substance, a higher dose is required subsequently to induce the same behavioral effect. Functional tolerance is due to neuroadaptations,[1,2] but the specific neurobiological mechanisms underlying tolerance are still poorly understood. Indeed, tolerance is understudied, despite being one of the criteria for diagnosing AUD and critical to the persistence of alcohol abuse.[3] A better understanding of the mechanisms of ethanol tolerance may lead to improved diagnosis and treatment of AUD.

Drosophila melanogaster is a proven useful model for studying the neurobiological and behavioral effects of alcohol. Indeed, flies display many of the behavioral responses observed in mammals, including humans, such as hyperactivity when exposed to low doses of alcohol and sedation with higher doses of alcohol. Flies, like humans, also show naïve avoidance of ethanol but learned preference upon repeated experiences, and they develop tolerance and experience withdrawal symptoms. Moreover, *Drosophila* share strong homology with human genes and conservation of signaling pathways and neurotransmitter systems, which allows genetic studies with high translational relevance for humans.[4–7]

Tolerance has been well-studied in *Drosophila*, and many tolerance mutants have been described (reviewed in References [5,8]). However, methods used to determine tolerance mutants have varied. For example, tolerance was initially described using an inebriometer for ethanol exposure. This device measures the ethanol-induced loss of postural control where flies that become sedated elute out the bottom of the inebriometer, are counted, and cease to be exposed to ethanol. By quantifying the relative increase in the mean time to elution between the first and second exposure, tolerance is determined.[1] In this approach, tolerance mutants are defined as flies with a significantly different percent increase in mean elution time from the genetic controls. It is worth noting that in the inebriometer, experimental and control flies may be exposed to a different dose of ethanol during the first, tolerance-inducing exposure. If experimental flies are resistant, for example, they may elute after 24 min of ethanol exposure, compared to 20 min for the control. Assuming similar ethanol absorption and metabolism (true for most published mutants), resistant flies will elute later, be exposed to more ethanol, but be exposed to the same 'effective dose', that is, the dose that causes complete loss of righting. However, other approaches to exposing flies to ethanol have been taken. Often, flies are exposed to ethanol vapor in closed containers which will not allow them to avoid exposure.[9] In such setups, unlike in the inebriometer, flies continue to be exposed to alcohol even after becoming sedated. Thus, experimental and control flies can be exposed for the same duration (i.e., dose), allowing for a 'fairer' comparison of tolerance; after all, the development of tolerance is dose-dependent.[1,10]

Previous research has sought to identify relationships between alcohol phenotypes. While ethanol tolerance and preference were found to be correlated, there was no relationship evident between initial resistance and tolerance phenotypes.[11] However, this was done in a fairly small sample. To determine whether the development of alcohol tolerance in any way depends on the initial resistance to the first sedation when the exposure dose is constant, we analyzed our own, and reanalyzed published tolerance data. We identified a highly significant inverse correlation between initial resistance (time to sedation) and tolerance (% increase in time to sedation). Our findings suggest that numerous genetic manipulations that, at face value, might be considered tolerance mutants may in fact be misclassified because they show initial sedation-sensitivity or sedation-resistance, which affects the development of tolerance secondarily. Knowing this inverse correlation also suggests that 'primary' tolerance mutants should be determined by correcting for this correlation, enabling the identification of mutants with tolerance phenotypes that are not solely the consequence of initial resistance phenotypes. We propose a predictive correlation function to aid in identifying *Drosophila* mutants that specifically affect the development of ethanol tolerance.

2 | MATERIALS AND METHODS

2.1 | Fly stocks and genetics

Fly rearing and crosses were done on a 12:12-h light–dark cycle on regular cornmeal/yeast/molasses food at 25°C/65% humidity (unless otherwise specified). *w*Berlin* flies were used as +/+ controls. Some defined mutant alleles flies were outcrossed for five generations to the *w*Berlin* genetic background. Other experimental flies (e.g., *UAS-RNAi* lines for knock-down) were compared to siblings that lacked the *UAS-RNAi* transgenic insert or to control flies that served to inject DNA to generate the *UAS-RNAi* line. Many fly strains were obtained from the Bloomington or Vienna stock centers or were generous gifts from colleagues. All fly lines used in this study are listed in Table S1.

2.2 | Behavioral experiments

3- to 7-day-old adult males were collected after eclosion and used for experiments after at least 16 h of recovery. Ethanol exposure and determination of ST-50 (time to half the flies being sedated) via measuring the flies' loss-of-righting reflex (LORR) was performed as described previously.[12] Briefly, flies were exposed to ethanol vapor and visually inspected every 2 min for LORR after light tapping, and the ST-50 for 8–10 flies was determined and counted for an *n* of 1. For tolerance, flies were exposed to ethanol for 22 min during first exposure and re-exposed 4 h later. Each set of experimental and control flies was assayed in parallel on the same day and repeated at least two times on different days. For Figures 4 and 6, flies were exposed to 1.5 times their ST-50, which was determined for each *n* of 1 (8–10 flies). Tolerance was calculated as the % increase in ST-50 from exposure 1 to exposure 2.

2.3 | Ethanol absorption

Ethanol concentration in flies was measured using the method established by Ishmayana et al.[13] Control and experimental flies (a total of $n = 10$ per condition were tested where $n = 1$ consisted of 10 flies) were exposed to ethanol vapor for 1.5 times the ST-50. At the end of the exposure, flies were frozen and homogenized. Two microliters of homogenates were used to measure ethanol concentration at 340 nm on a Nanodrop 2000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). Reagents used for this assay were Alcohol Dehydrogenase (Cat No J65869.9+, lot S301038, Thermo Fisher Scientific, Waltham, MA, USA) and beta nicotinamide adenine nucleotide (Cat No J62337.03+, lot M251003, Thermo Fisher Scientific, Waltham, MA, USA).

2.4 | Experimental design and statistical analyses

Data are represented by mean ± SEM for ethanol sedation and tolerance data. Data were analyzed using GraphPad Prism Software version 10.1.1 for MacOS (GraphPad Software, Boston, MA, USA, www.graphpad.com), and statistical analysis was done with unpaired Student's *t*-test. We then calculated the effect sizes with Hedge's *g* for ST-50 and % tolerance (± 95% confidence intervals for some panels). We chose to calculate effect sizes because they are standardized across studies, allowing for straightforward comparisons of data from different sources.[14] We plotted the effect size for ST-50 on the *x*-axis and the tolerance effect size on the *y*-axis and performed a simple linear regression. We also determined ST-50s and tolerance from previous published studies.[15–22] Our inclusion criterion was that the control and experimental flies were subject to the same dose of ethanol for the same period of time. Therefore (except for Figure 5), we did not analyze experiments done using an inebriometer, where the exposure ends as soon as flies become sedated. This results in different exposure times for different flies (such as experimental vs. controls) and also distinct exposure doses. The reasons for this are expanded upon in Section 3.6 (Figure 8). For previously published data (except for Figure 5), we included two experimental manipulations per gene, often using a classical mutant and an RNAi-mediated knockdown when available. The specific experimental manipulations for each gene analyzed and the figures in which they appear are available in Table S1.

We performed a Monte Carlo simulation of the linear regression for tolerance effect size as predicted by initial resistance effect size using a custom R script. For each of the 74 genes in our pooled analysis (Figure 7A), we randomly selected one genetic manipulation which we used as the input to the Monte Carlo simulation. We iterated the simulation 1000 times, generating a distribution of R^2 and slope values.

3 | RESULTS

3.1 | Inverse correlation of initial resistance and tolerance phenotypes in our mutants

Having examined numerous genes for their alcohol phenotypes over the years, we have sometimes noticed that mutants that were resistant to ethanol-induced sedation also seemed to develop less tolerance upon a second exposure. In a previous study, we explicitly tested for a relationship between the levels of resistance to naïve alcohol-induced sedation (henceforth referred to as initial resistance) and the levels of alcohol-induced tolerance in a family of jumonji-domain histone demethylase (*HDM*) mutants and found no correlation.[23] We re-examined these data and calculated the effect sizes compared to wild-type for ethanol resistance, as measured by the ST-50, the time to sedation for half of the flies. An effect size >0 signifies increased initial resistance (Figure 1A). We also determined effect sizes for tolerance, as measured by the percentage increase in ST-50 from the first to the second exposure. As before, an effect size >0 signifies the development of more tolerance (Figure 1A). Effect sizes are useful in the context of analyzing data from various sources because they are standardized and enable a meaningful comparison of data that may have originally been reported on different scales. In the experiments we analyzed, experimental genotypes and control flies were exposed to the same dose of alcohol (i.e., alcohol concentration*duration of exposure). Like in our previous study, when re-evaluated the data for effect size, there was no correlation between effects on initial resistance and tolerance for the 13 loss-of-function (LOF) *HDM* mutants (Figure 1B). However, of these 13 *HDM* mutants, 7 did not show any significant phenotypes to begin with, that is, a change from the wild-type control in initial resistance and/or tolerance[23] (Figure 1B; gray points). To ask whether there was a correlation between initial resistance and tolerance phenotypes, we therefore focused our analysis on the 6 *HDM* mutants that did show a significant change from the wild-type control in initial resistance and/or tolerance.[23] We noticed that 5 of the 6 mutants lay in effect size quadrants where increased initial resistance correlates with decreased tolerance and vice versa—the one exception being $Kdm3^{KO}$ mutants, which show less initial

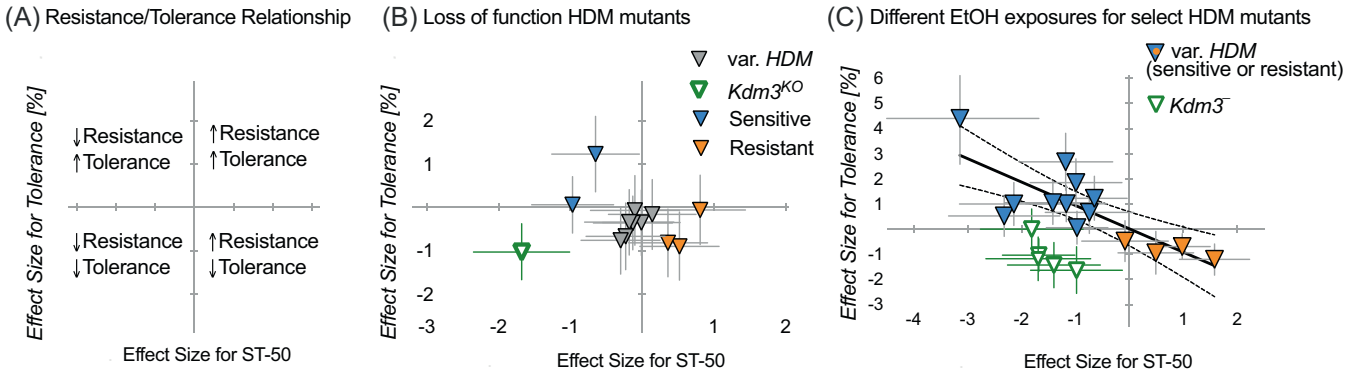


FIGURE 1 (A) Plot of effect sizes for experimental versus control phenotypes of sedation (ST-50) on *x*-axis and effect sizes for tolerance on *y*-axis. Data points in the upper left quadrant show decreased initial resistance during the first exposure with increased tolerance after the second exposure, decreased resistance and decreased tolerance for lower left quadrant, increased resistance and decreased tolerance for the lower right quadrant and increased resistance with increased tolerance for the upper right quadrant. (B) Effect sizes for tolerance and for sedation for loss-of-function histone demethylase (HDM) mutants were plotted. Gray symbols depict HDM mutants with no resistance or tolerance phenotype (*JMJD4*, *KDM4A*, *Jarid2*, *KDM4B*, *PSR*, *KDM2*, and *JMJD5*). Blue symbols depict initially sensitive mutants (*No66A* and *lid*), orange symbols resistant mutants (*UTX*, *HSPBAP1*, and *JMJD7*), and green is a *Kdm3* mutant, a possible exception to the inverse correlation.[23] Here, and in subsequent panels, effect size error bars are 95% confidence intervals. (C) Effect sizes for tolerance and for sedation assays for loss-of-function HDM mutants that showed a phenotype previously (in B). These mutants were exposed to different alcohol concentrations.[23] There is a significant inverse correlation between initial resistance and tolerance with these mutants when *Kdm3* is excluded. The linear regression plotted was calculated excluding *Kdm3* data points; however, even with *Kdm3* data included, there is a significant correlation (see text for details). Mutants include knock outs and neuron-specific knock-down by RNAi.[23]

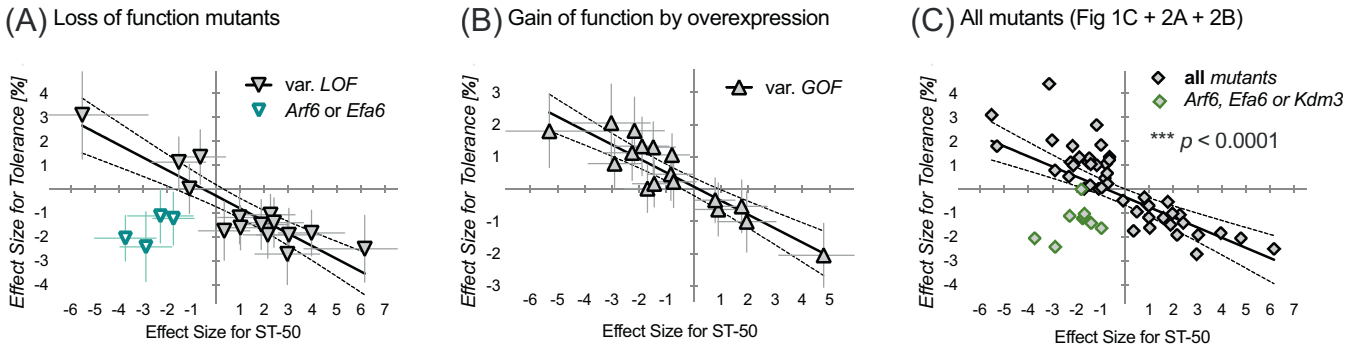


FIGURE 2 (A) Effect sizes for tolerance and for sedation for various of loss-of-functions (LOF) mutants from our lab, including *Arf6* and *Efa6* mutants, were plotted (unpublished data).[24,34,42,50] Teal symbols depict *Arf6* and *Efa6* mutants, which are exceptions to the inverse correlation based on effect size quadrant. The linear regression was calculated excluding Arf6 and Efa6 data points. Inclusion of these mutants still resulted in a significant correlation (see text). (B) Effect sizes for tolerance and for sedation for various gain-of-function (GOF) neuronal manipulations were plotted. The linear regression was calculated including all data points. (C) Effect sizes for tolerance and for sedation for all of our mutants (data from Figures 1C and 2A,B) were plotted. The linear regression was calculated including all data points. Exceptions to the inverse correlation based on effect size quadrant (*Kdm3*, *Arf6*, *Efa6*) are depicted by a green symbol, but are included in the correlation calculation.

resistance and develop less tolerance (Figure 1B). Our previous study followed up on the 4 of these 6 mutants that had large effect sizes by performing additional experiments at different exposure doses (ranging from low to high EtOH concentrations).[23] We also reanalyzed this data for effect size. The 3 non-*Kdm3* mutants showed an obvious inverse correlation between initial resistance and tolerance phenotypes ($R^2 = 0.63$, F (1, 12)=20, $p = 0.0008$; Figure 1C). Even including *Kdm3*, there was a significant correlation ($R^2 = 0.25$, F (1, 17) = 5.6, $p = 0.031$).

To test the hypothesis that there is an inverse correlation between initial resistance and tolerance phenotypes more generally, we examined additional genetic manipulations that caused significant initial resistance and/or tolerance phenotypes when experimental and control flies were exposed to the same dose of alcohol. First, we analyzed loss-of-function (LOF) mutants in 12 non-*HDM* genes we have studied over the years (most published, but some unpublished), and 10 showed an inverse correlation between resistance and tolerance effect sizes ($R^2 = 0.80$, F (1, 13) = 52, $p < 0.0001$; Figure 2A). The

two exceptions to the inverse correlation were mutants in *Arf6* and its activator *Efa6*, which both showed reduced initial resistance and reduced tolerance (Figure 2A), similar to *Kdm3* (Figure 1B). The correlation was significant even including *Arf6* and *Efa6* ($R^2 = 0.32$, F (1, 17) = 8.1, $p = 0.011$). Including the above *HDM* mutants, of the 16 genes examined, 13 LOF mutants showed inversely correlated effects on initial resistance and tolerance.

We also analyzed several gain-of-function (GOF) mutants, using the *Gal4/UAS* system to overexpress wild-type or constitutive-active versions of various genes in a neuron-specific manner (i.e., in all neurons or in subsets of neurons such as GABAergic or cholinergic neurons). We analyzed 7 genes in 17 experiments, using 9 different *Gal4* drivers, which also showed a significant inverse correlation between their GOF initial resistance and tolerance phenotypes ($R^2 = 0.81$, F (1, 15) = 65, $p < 0.0001$; Figure 2B). Taken together, the combined 55 experiments shown in Figures 1 and 2, manipulating 21 different genes, showed an inverse correlation with an R^2 of 0.4 and $p < 0.0001$ (F(1,53) = 35; data from Figures 1C and 2A,B, presented together in Figure 2C).

3.2 | Inverse correlation of initial resistance and tolerance phenotypes in other published mutants

Since all data in Figures 1 and 2 were generated in our own lab, we wondered whether our observed inverse correlation would hold true for published phenotypes from other labs. We combed the literature and analyzed publications from the last 15 years that determined initial resistance and tolerance similarly to the experiments conducted in our lab. The main inclusion criteria were that: a) experimental and control flies were exposed in parallel and b) they were exposed to the same dose of ethanol. By necessity, this excludes any experiments done in an inebriometer, where the exposure ends as soon as flies become sedated, meaning that experimental and control flies receive different doses of ethanol, if experimentals show an initial resistance phenotype. This left us with 8 papers from 6 labs describing 28 manipulations in 16 genes and their resultant ethanol-related phenotypes. One of these genes (*mys*) was also tested in our lab's studies described in Figure 2 (see Table S1 for more information).[15,24] We analyzed published mutants with significant initial resistance and/or tolerance phenotypes, again revealing a significant inverse correlation between initial resistance and tolerance (Figure 3A; $R^2 = 0.57$, F (1, 26) = 35, $p < 0.0001$). Together, these analyses illustrate that in our hands and other labs, there is a significant inverse correlation between initial resistance and relative tolerance when experimental and control flies are exposed to the same dose of alcohol.

Devineni and colleagues[11] had also previously asked whether initial resistance and tolerance phenotypes correlate, analyzing 18 mutants in various genes from a large forward genetic screen for alcohol-response phenotypes. Two of these genes were also tested in our lab's studies described in Figure 1A (*Kdm2*) and 2A,C (*RhoGAP18B*; see Table S1 for more information).[23,25] Unlike in our analysis, where tolerance was calculated as relative (percent increase from exposure 1 to exposure 2), they measured tolerance as an absolute increase in minutes from exposure 1 to exposure 2. In doing this, they found no correlation between initial resistance and tolerance. This data, replotted for effect sizes, is shown in Figure 3B ($R^2 = 0.006$, F (1, 16) = 0.1, $p = 0.75$; not significant, as published by Reference [11]). Using these data, we re-calculated tolerance as a percent increase relative to the initial resistance measured by the initial ST-50 (as in Figures 1 and 2) and plotted the resulting effect sizes. When measured this way, there was again a significant inverse correlation between initial resistance and tolerance (Figure 3C; $R^2 = 0.42$, F (1, 16) = 12, $p = 0.0035$).

3.3 | Tolerance should be expressed in relative, not absolute, terms

As outlined above, we determined a significant inverse correlation between initial resistance and tolerance phenotypes when tolerance is expressed as a fractional change relative to the first ST-50. While

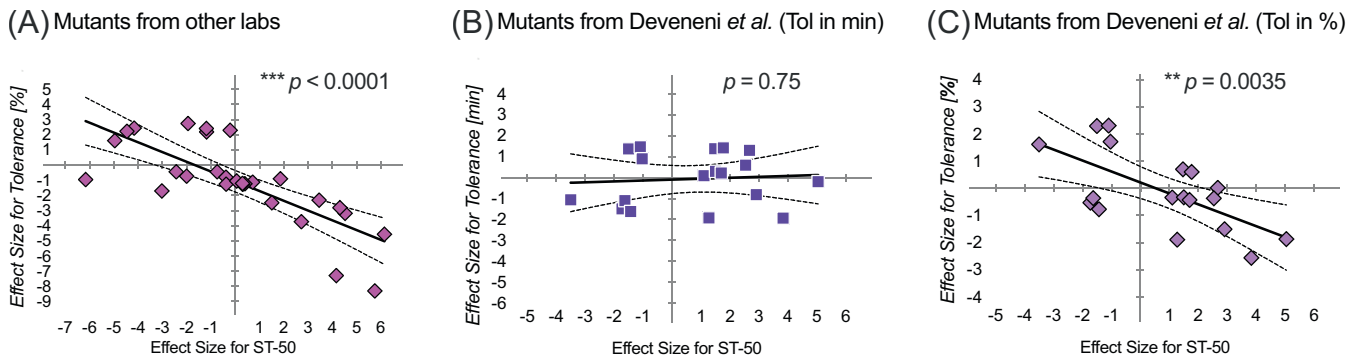


FIGURE 3 (A) Effect sizes for tolerance and for sedation for various mutants published by other labs.[15–17,19–22,31] The linear regression was calculated including all data points. (B) Effect sizes for tolerance and for sedation for mutants published in Devineni et al.,[11] where tolerance is calculated in absolute terms, that is, difference in minutes. The linear regression was calculated including all data points. (C) Effect sizes for tolerance and for sedation (ST-50) for mutants shown in B, where tolerance is re-calculated in relative terms, that is, % increase in ST-50. The linear regression was calculated including all data points.

some labs also calculate tolerance this way, other publications, including the one described in Figure 3B,C,[11] express tolerance as an absolute difference in minutes, subtracting the initial ST-50 from the second ST-50.[26] Since the inverse correlation that we found holds true for tolerance analysis in relative terms (see Figure 3B vs. Figure 3C), we wanted to determine which of the two measures is more accurate for analyzing tolerance.

Increasing the dose (i.e., duration) of the first ethanol exposure increases tolerance.[1] This increase in tolerance is reflected in both relative and absolute terms and is therefore not helpful in determining the best way to calculate tolerance. However, we reasoned that when flies of the identical genotype are exposed to the same initial dose of ethanol, tolerance should be the same. We therefore exposed *w*Berlin* flies to two different concentrations of ethanol (95% vs. 80%), where flies receiving the higher concentration were exposed for a shorter time than flies receiving the lower concentration (Figure 4A). In both cases, flies were exposed to 1.5 times the ST-50, which we independently determined for each vial of flies tested, resulting in complete loss of the righting reflex. During both the first and second exposure, the ST-50 was significantly shorter in the 95% EtOH-exposed flies (8.9 min on the first exposure and 11.5 min on the second exposure) than in the 80% EtOH-exposed flies (12.4 min on the first exposure and 16.4 min on the second exposure; Figure 4B). Importantly, these two exposure paradigms resulted in indistinguishable internal ethanol concentrations after the first exposure (t (18) = 0.6, $p = 0.55$; Figure 4C), suggesting that these flies indeed received the same exposure dose. We then quantified the tolerance these two exposures induced in both relative and absolute terms. The amount of tolerance was significantly different when calculated in absolute terms as minutes ($p = 0.040$, t (22) = 2.2; Figure 4D), while it was not significantly different when expressed in relative terms, as percent increase ($p = 0.40$, t (22) = 0.4; Figure 4E). These data show that our two paradigms of exposing flies to ethanol resulted in the same initial exposure dose (as measured by internal ethanol concentration after exposure 1). Behaviorally, they gained the same amount of tolerance in percent, while tolerance expressed in absolute minutes was significantly different. Given that the same genotype of flies received the same dose of ethanol in both exposures, our data indicate that ethanol's effect on tolerance should be expressed fractionally relative to the initial resistance. These data also support our finding of an inverse correlation between initial resistance and tolerance phenotypes.

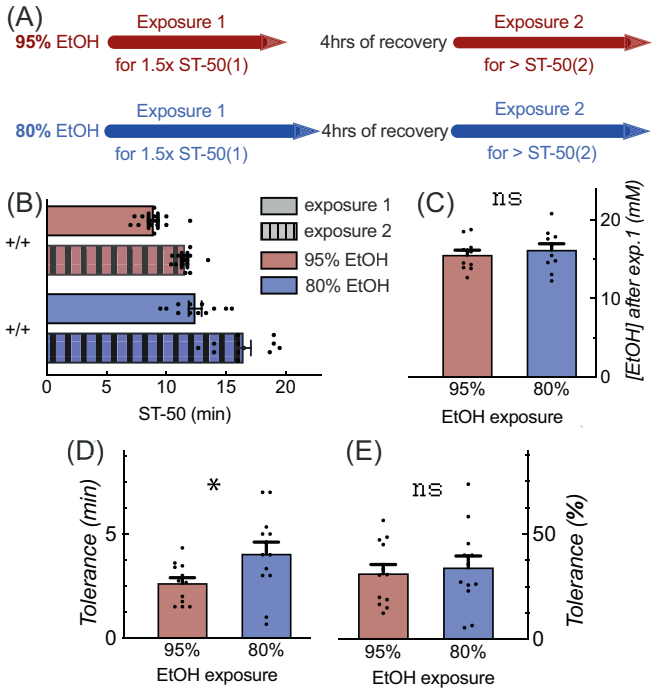


FIGURE 4 (A) Schematic of EtOH exposure paradigms to determine if tolerance should be calculated in minutes or % increase. The same genotype, *w*Berlin*, was exposed in the two different ways shown. (B) ST-50s of first and second exposures for flies exposed to 95% EtOH (brown) and 80% EtOH (blue), $n = 12$ each. Data in this figure are shown as mean ± SEM. (C) Internal ethanol concentrations (mM) in flies collected after the first exposure to either 95% or 80% EtOH. (D, E) Tolerance developed in flies exposed to either 95% or 80% EtOH expressed in absolute terms (D, difference in minutes) and in relative terms (E, % increase).

3.4 | Additional evidence to support an inverse correlation

In Figure 3, we examined published mutants to verify our finding of an inverse correlation. In our literature search, we identified another publication that tested 205 wild-type *Drosophila* Genetic Reference Panel (DGRP) lines for ethanol resistance and tolerance.[27] In this paper, flies are tested in the inebriometer, and it therefore did not meet our inclusion criteria since the exposure ends as soon as flies become sedated, causing experimental and control flies to receive different doses of ethanol. Nevertheless, because it is a large data set that tests our phenotypes of interest, we decided to assess any correlation between initial resistance and tolerance. Using the provided raw data on the mean elution time (MET) for the first and second exposure for each of the 205 lines (their Additional File #2), we calculated tolerance as a percent increase from the first to second exposure (Figures 3C and 4E). We were unable to calculate effect sizes for these data because of the N for each line being too small, so we plotted the initial MET against the percent tolerance and obtained a significant inverse correlation in both males (Figure 5A; $R^2 = 0.29$, F (1,203) = 85, $p < 0.0001$) and females (Figure 5B; $R^2 = 0.246$, F (1,202) = 170, $p < 0.0001$). Therefore, despite methodological differences in data collection in this study compared to the ones we evaluated, we still found a strong inverse correlation between ethanol resistance and tolerance phenotypes in this large analysis of 205 fly lines.

Given our determination that expressing tolerance relative to initial resistance is an accurate measure and that there is a significant inverse correlation between these two, we were interested in further expanding our analysis. In addition to examining various Mendelian mutants (Figures 1–3), we have also used RNA-interference (RNAi) to knock down the expression of many genes.

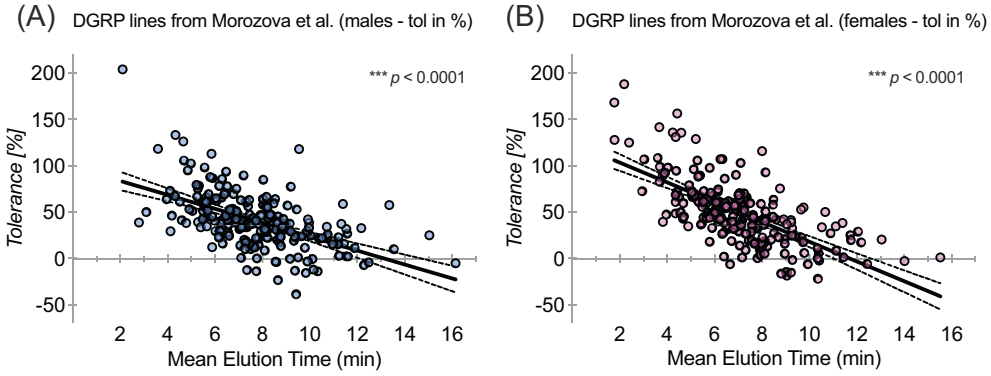


FIGURE 5 Mean elution time (MET) versus percent increase in tolerance for 205 DGRP lines from Morozova et al.[27] Males (A) and females (B) both have a significant inverse correlation between MET and % tolerance. The linear regression was calculated using all data points. Note that unlike other experiments shown in this paper, these data were acquired in an inebriometer.

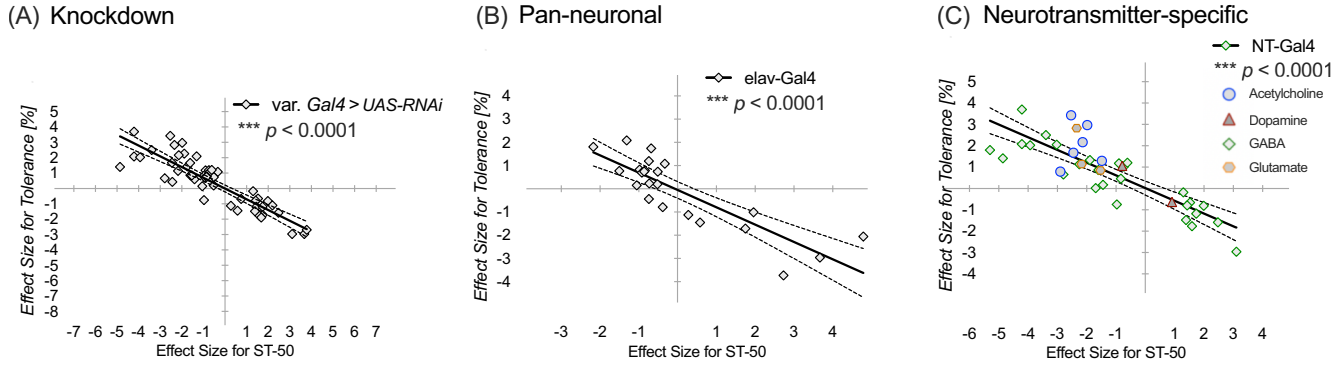


FIGURE 6 (A) Effect sizes for tolerance and for sedation for various Gal4-mediated knockdown experiments performed in our lab. The linear regression was calculated including all data points. (B) Effect sizes for tolerance and for sedation for all pan-neuronal (*elav-Gal4*) manipulations (data are from Figures 2B, 3A, and 6A). The linear regression was calculated using all data points. (C) Effect sizes for tolerance and for sedation for neurotransmitter-specific manipulations (acetylcholine in blue, dopamine in red, GABA in green, glutamate in orange). Data are from Figures 2B and 6A. The linear regression was calculated using all data points.

We collated all the data for these experiments, again analyzing experiments run in parallel with identical ethanol exposures where experimental flies showed a significant difference in initial resistance and/or tolerance from the controls. Here, we note that we have not verified the knock-down efficiency of the RNAi transgene for most of these experiments, nor have we ruled out any possible off-target effects. We therefore do not claim that the presumed target genes are responsible for the observed phenotypes. However, we stress that experimental and control flies were exposed simultaneously, in parallel, to identical ethanol concentrations and durations and that they are genetically distinct from each other and showed significant resistance and/or tolerance phenotypes. Therefore, these experiments are well-suited to answer whether genetic differences cause correlated ethanol response phenotypes in initial resistance and tolerance. We found that in 52 additional experiments, with 32 distinct RNAi-transgenes (putatively targeting 23 genes), using 10 different *Gal4* drivers, there was also a significant inverse correlation between initial resistance and tolerance ($R^2 = 0.78$, F (1, 50) = 177, $p < 0.0001$; Figure 6A). We then combined the analyses from Figures 2C, 3A,C, and 6A and assessed the relationship between initial resistance and tolerance based on *Gal4* driver. All combined pan-neuronal manipulations (knockdown and overexpression) again revealed a strong inverse correlation, ($R^2 = 0.72$, F (1, 21) = 55, $p < 0.0001$; Figure 6B) as did neurotransmitter-specific knockdown and overexpression ($R^2 = 0.69$, F (1, 36) = 83, $p < 0.0001$; Figure 6C).

3.5 | An unbiased analysis supports our finding of an inverse correlation

Given the strong inverse correlation between initial resistance and tolerance phenotypes that we have shown thus far, we combined the analyses from Figures 2C, 3A,C, and 6A into a single plot (Figure 7A) which showed a significant inverse correlation across all experiments and manipulations ($R^2 = 0.51$, F(1,156) = 161, $p < 0.0001$; all data included, except Figure 5). Figure 7A shows 158 data points accounting for manipulations targeting 74 different genes, meaning that some genes are represented more than others in this plot. We set out to study the relationship between phenotypes and were less concerned with the specific genetic manipulations assessed, but we recognized the possibility of biasing our data toward overrepresented genes and

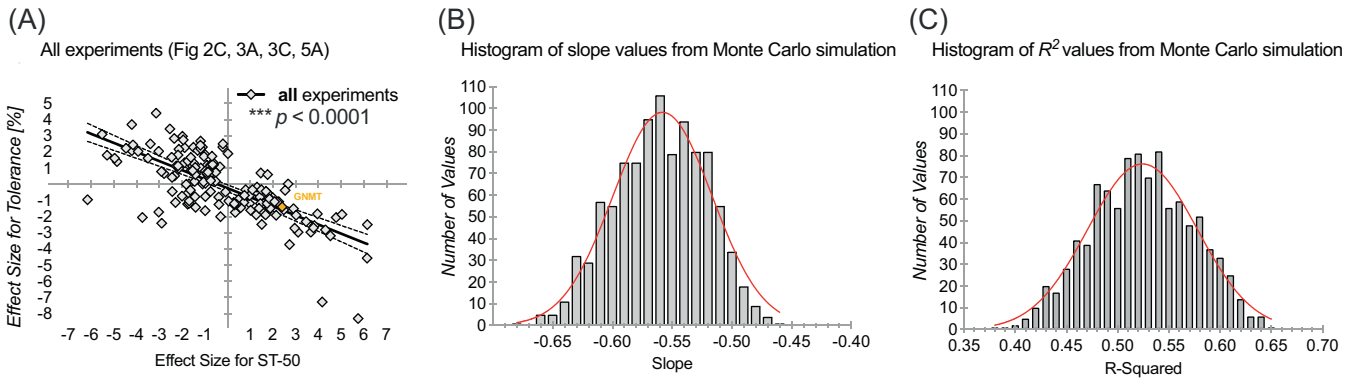


FIGURE 7 (A) Effect sizes for tolerance and for sedation (ST-50) for all of our mutants (data from Figure 2C, 3A,C and 6A). The linear regression was calculated including all data points. *GNMT* mutant is highlighted in yellow. (B) Histogram of the slope values generated by the Monte Carlo simulation, picking one manipulation for each gene from panel A. There are 1000 values in bins of 0.01. Gaussian curve shown in red. (C) Histogram of the R^2 values generated by the Monte Carlo simulation. There are 1000 values in bins of 0.01. Gaussian curve shown in red.

wanted to control for this scenario. To do so, we randomly selected a single genetic manipulation for each of the 74 genes in Figure 7A and used the initial resistance and tolerance effect sizes for this manipulation as inputs into a Monte Carlo simulation for the linear regression. This type of simulation is an unbiased approach that utilizes repeated random sampling to generate dependent variables based on a given input distribution.[28] Once a pair of input effect sizes was determined for each of the 74 genes, the Monte Carlo simulation determined a linear regression based on these 74 pairs of effect sizes, and repeated the selection/analysis steps 1000 times. The result was a distribution of 1000 slope (Figure 7B) and R^2 (Figure 7C) values. Both of these measures were tightly distributed (slopes: mean = −0.56, SD = 0.03; R^2: mean = −0.52, SD = 0.05), and the overall linear model had a highly significant *p*-value of 4.8*10e−12. Therefore, our Monte Carlo analysis supports our finding of a significant inverse correlation between initial resistance and tolerance phenotypes, since it represents an unbiased approach that eliminates possible overrepresentation of some genes.

3.6 | Tolerance phenotypes can be a consequence of initial resistance phenotypes

Our inverse correlation shows that flies that are more sensitive to ethanol (i.e., become sedated quickly) generally develop more tolerance, and resistant flies less. One explanation for this is that highly sensitive flies that are exposed for the same duration as less-sensitive controls achieve a greater depth of sedation which facilitates the development of more tolerance. Therefore, it may be the case that the absolute dose in mM ethanol does not matter as much for the homeostatic process of tolerance but that, instead, the depth of sedation and with it, the effectiveness of depression of CNS activity, determines tolerance. Indeed, longer first ethanol exposures are known to induce more tolerance,[1,10] presumably because they depress CNS activity more strongly and for a longer duration.

What implications does this have when determining tolerance for a mutant that affects initial resistance? Since exposing a given mutant and its control to the same dose may result in a distinct depth of sedation/effectiveness of CNS depression, we reasoned that they should be exposed to the same 'effectiveness of intoxication,' most easily measured by the behavioral effect on sedation. For example, in the inebriometer, flies are exposed to complete loss-of-righting, fall through the exposure column, and are no longer exposed. Thus, experimental and control flies are exposed to the same behavioral effect, or effectiveness of intoxication. If this complete loss-of-righting induced distinct amounts of tolerance, then such flies are 'primary tolerance mutants'. Conversely, when sedation and tolerance effects lie on the inverse correlation curve (of identical exposures), flies are tolerance mutants secondarily due to distinct levels of effectiveness of sedation during the first exposure, thus classifying them as 'secondary tolerance mutants'. GNMT is an enzyme involved in the 1-Carbon cycle, and when exposed for the same duration as controls (Figure 8A), *GNMT* LOF causes increased initial resistance (t (21) = 4.0, p = 0.0007; Figure 8B) and reduced tolerance (t (20) = 3.8, p = 0.0012; Figure 8C). At face value, these data suggest that *GNMT* is a tolerance mutant. However, the tolerance phenotype was exactly as predicted by the inverse resistance/tolerance correlation (see Figure 7A). We therefore predicted that if we exposed wild-type and *GNMT* mutants to the same depth of sedation—1.5 times their respective initial ST-50—they would both develop the same amount of tolerance. Indeed, exposing *GNMT* mutants and wild-type to ethanol for 1.5*ST-50 necessitated longer initial exposures in *GNMT* mutants (15.5 min vs. 12 min for wild-type; Figure 8D). Although there was still a significant difference in initial resistance (t (21) = 3.2, p = 0.0004; Figure 8E), there was no difference in the subsequent development of tolerance (t (21) = 1.3, p = 0.20; Figure 8F) using this exposure paradigm. Therefore, *GNMT* may at first appear to be a tolerance mutant due to its development of significantly lower tolerance when exposed to the same dose as wild type, but we suggest that in this exposure paradigm, *GNMT*'s tolerance effects are simply a secondary

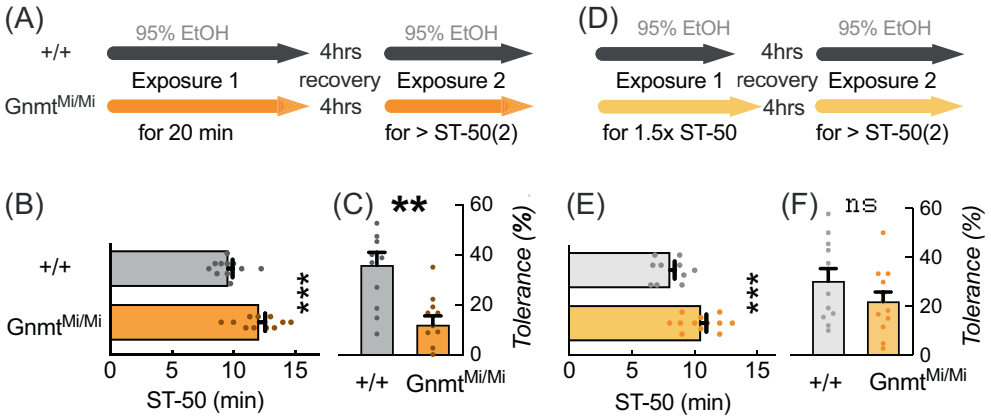


FIGURE 8 (A, D) Schematic of EtOH exposure paradigm of *GNMT* mutant and control flies. (B, E) Ethanol sedation phenotype for controls versus *GNMT* mutants during regular exposure paradigm (A, B) or when exposed to 1.5*ST-50 (D, E). In both cases, *GNMT* mutants are resistant to the initial ethanol-induced sedation. Data shown as mean ± SEM. (C, F) Tolerance (% increase) phenotype for controls versus *GNMT* mutants during regular exposure paradigm (C) or when exposed to 1.5*ST-50 (F).

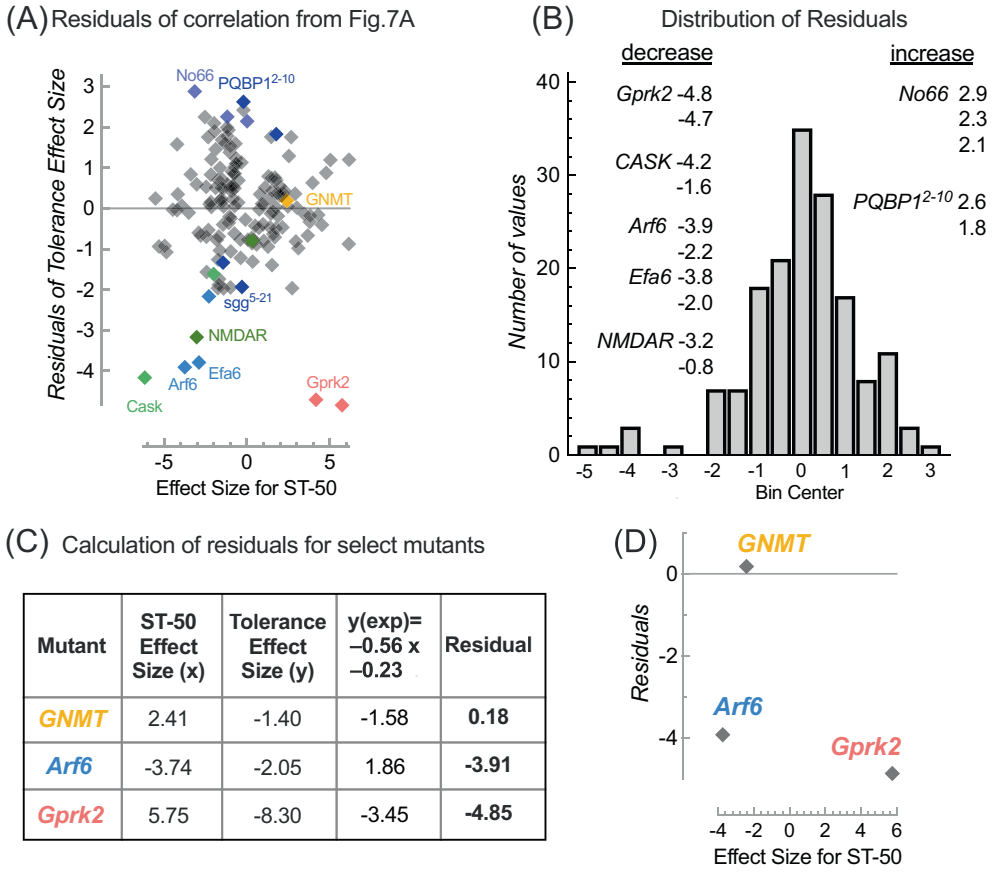
consequence of the initial resistance. Indeed, when *GNMT* mutants and wild-type flies receive an amount of alcohol that induces equivalent behavioral effects, the tolerance phenotype is lost. Knowing the inverse correlation of alcohol resistance and tolerance therefore alters the interpretation of the initial *GNMT* tolerance phenotype.

4 | DISCUSSION

Here, we analyzed a large collection of genetic manipulations that result in ethanol resistance and/or tolerance phenotypes and detected a significant inverse correlation of the two measures when both experimental and control flies were exposed to the same initial, tolerance-inducing ethanol dose. Mutants with increased initial resistance developed less tolerance, and ones with decreased initial resistance developed more tolerance. One possible explanation of this inverse correlation is that apparent tolerance phenotypes arise from more sensitive mutants because they become more deeply sedated (and for a longer duration) after the first exposure, and they therefore develop more tolerance. This interpretation is consistent with published findings showing that larger exposure doses induce more tolerance.[1,10] Conversely, mutants that are resistant to ethanol are only lightly sedated at the end of the exposure compared to controls and therefore develop less tolerance. The inverse correlation may reflect the homeostatic control of CNS activity. If tolerance is caused by the upregulation of neuronal activity triggered by prior ethanol-induced depression of neuronal activity,[8] then the extent of tolerance developed may reflect how effective/deep the initial ethanol-induced neuronal depression was for a given first ethanol dose. This would support the idea that the functional effect of ethanol on the neurons likely matters more than the actual dose of ethanol. Thus, when the tolerance-inducing mechanism is intact, slight neuronal depression in resistant mutants and strong neuronal depression in sensitive mutants will induce slight or strong tolerance, respectively. This relationship would result in our observed inverse correlation and suggests that many mutants develop tolerance as a function of their initial resistance, which we categorize as secondary tolerance mutants. Therefore, primary tolerance mutants will show resistance and tolerance effects that do not lie on the inverse correlation curve but will instead be far away from that curve.

The distance from the inverse correlation curve is reflected by the residuals (Figure 9A). Figure 9B shows the distribution of residuals from this correlation, which could be used as a guide to determine which mutants are primary tolerance mutants and which ones to follow up on to investigate tolerance mechanisms. These residuals can be considered 'corrected' effect sizes for the tolerance phenotypes. Classically, effect sizes of ±0.8 or higher/lower are considered strong, which would apply to 70 out of 158 residuals. For the initial resistance phenotypes, 127 out of 158 data points lie beyond ±0.8. This suggests that many more of our analyzed genotypes are strong initial resistance mutants than are strong tolerance mutants, consistent with our finding that tolerance is secondarily affected by initial resistance. This also raises the question: where should the tolerance residual cutoff be to consider a mutant a primary tolerance mutant? This cutoff is in the eye of the beholder, and from our experience, we prefer to work with strong mutants that have effect sizes beyond ±1.2. This would hold true for 45 tolerance residuals. However, because tolerance data are more variable than initial resistance data, we prefer an even larger effect size of ±1.5, which is the case for 36 tolerance residual data points. Regardless of the exact threshold chosen, in Figure 8, we show that *GNMT* is not a primary tolerance mutant since its tolerance phenotype disappears when mutants are exposed to the same depth of sedation as controls. Consistent with this, the residual for *GNMT* is 0.18 (Figure 8A, C-D), indicating that this mutant develops tolerance as expected from the inverse correlation and is therefore not a primary tolerance mutant.

The residual plots (Figure 9A,B) highlight a few strong primary tolerance mutants. Among the strong tolerance mutants are mutants



Mutant	ST-50 Effect Size (x)	Tolerance Effect Size (y)	y(exp)= –0.56 x –0.23	Residual
GNMT	2.41	-1.40	-1.58	**0.18**
Arf6	-3.74	-2.05	1.86	**-3.91**
Gprk2	5.75	-8.30	-3.45	**-4.85**

FIGURE 9 (A, B) Residual plots for all the data ($n = 158$). Residuals (*y*-axis) in the XY plot (A) indicate how far from the inverse correlation curve the data points lie, while the frequency plot (B) shows a normal distribution of the residuals centered around 0 (mean = 0.004). Some potential strong, primary tolerance mutants are highlighted (*Arf6*, *Efa6*, *CASK*, *NMDAR*, *Gprk2*, *No66*, *PQBP1*$^{2-10}$ and *sgg*$^{5-21}$) as well as *GNMT* (yellow), which is not a primary tolerance mutant. (C) Example of how to calculate residuals using the equation $y(\text{exp}) = -0.56\ x - 0.23$ with *GNMT*, *Arf6* and *Gprk2* data, where y(exp) stands for the tolerance effect size (*y*) expected. (D) Plot of residuals calculated in C versus effect size for ST-50.

PQBP1$^{2-10}$ and *sgg*$^{5-21}$ from Devineni et al.[11] In one of two experiments, these showed the same initial ST-50 as the control and then developed more or less tolerance, respectively. By definition, such mutants are primary tolerance mutants since a phenotype only occurs for tolerance but not for initial resistance. Furthermore, they are also predicted to be primary tolerance mutants by our correlation, since our curve is close to the origin of the plot ($x = y = 0$), and residuals at $x = 0$ (i.e., no initial resistance phenotype) equal the measured tolerance effect size *y* (see Figures 7A and 9A). *Kdm3* mutants show reduced initial resistance and reduced tolerance (counter to the prediction from the inverse correlation), suggesting that *Kdm3* might be involved in both setting initial neuronal activity and in the mechanism of increasing neuronal activity after the initial sedating ethanol exposure. Conversely, the *HDM* mutant *No66* develops more tolerance than expected in 3 of 6 experiments. Interestingly, long exposures at low ethanol concentrations exacerbated tolerance strongly, while short, high-concentration exposures caused small effects on tolerance. This suggests that some mutant phenotypes are dependent on the kinetics of the exposure, but we did not observe this kinetic-dependent tolerance phenotype for other *HDM* mutants like *Kdm3*.[23]

Mutants with residuals far from zero provide potential insights into the mechanisms of alcohol tolerance. The residuals plotted as a histogram (Figure 9B) show a cluster of 6 data points with residuals of −3 or lower, suggesting that these mutants' phenotypes reflect strong primary tolerance mutants that develop little tolerance. These mutants include the genes encoding Gprk2, a G protein-coupled kinase,[22,29] CASK, a member of the MAGUK family of scaffolding proteins,[17] the NMDA Receptor, which interacts with CASK,[30–32] and Arf6, a small GTPase involved in membrane trafficking and actin remodeling and its activator Efa6.[33–35] Mutants for Arf6 and Efa6 are highly sensitive to ethanol-induced sedation but induce very little tolerance.[35] We have placed Arf6 downstream of the insulin receptor (InR) and upstream of S6 kinase (S6k) in a pathway mediating initial resistance to ethanol. Overexpression of Arf6, as well as constitutive-active InR and S6k, cause increased initial resistance.[36] Similarly, the loss of ArfGAP3, an inactivator of Arf6, also causes increased initial resistance. All of these manipulations lead to decreased tolerance (in percent, relative to the first exposure), but not more so than expected based on their initial resistance phenotypes. In other words, these manipulations cause phenotypes consistent with our described inverse correlation. This suggests that the activity of this pathway is necessary for the development of tolerance, while gains of function in this pathway are not sufficient to cause excessive tolerance. Similarly, mutants for RhoGAP18B and Rsu1, both negative regulators of Rho GTPases, affect initial resistance but not tolerance.[24,25] Mutations upstream of RhoGAP18B and Rsu1 in *mys*, encoding the cell adhesion molecule ß-integrin, are also initially sensitive but develop tolerance as expected (by the inverse correlation).[15] This suggests that while both the Rho-family and Arf6 GTPase pathways are involved in setting initial resistance to ethanol exposure, only the Arf6 pathway is critical for ethanol-induced tolerance.

Although fewer data points have highly positive residuals, indicating the development of more tolerance than controls, mutant *PQBP1*$^{2\text{-}10}$.[11] and *No66*[23] have residuals greater than 2. For *No66*, effects are dose-dependent, where a low ethanol concentration suggests that No66 is a tolerance mutant while a high concentration does not. The relative lack of mutants which confer greater tolerance suggests that the biological mechanism of tolerance is more easily disrupted than potentiated. This might make sense in the context of a neuronal homeostasis model for ethanol tolerance. Since high doses of alcohol cause CNS depression, increased tolerance could theoretically be facilitated by a mutation that causes increased excitability in excitatory neurons, dampening the CNS-depressing effects of alcohol. In this model, these same mutants would also necessarily have an initial resistance phenotype, as if they were 'pre-tolerant.' However, it is likely that because of this initial resistance phenotype, one would see very little development of tolerance since the biological process by which tolerance occurs is already potentiated (akin to our observed negative correlation between initial resistance and tolerance). Similarly, if a primary tolerance mutant would be unable to increase excitability in excitatory neurons as a function of a CNS-depressing alcohol exposure, that mutant may have less excitable excitatory neurons to begin with and might therefore have an initial sensitivity phenotype. Together, this might explain why we find more datapoints in the lower left quadrant (less resistance, less tolerance), than in the upper right quadrant (more resistance, and more tolerance). We focus here on rapid tolerance, which is the tolerance to ethanol gained between separate exposure sessions, since acute tolerance, which is the tolerance to ethanol gained within a single exposure,[37] is rarely studied in *Drosophila*. Mechanisms of acute tolerance are also not well-understood but given evidence of genetic mediation of acute tolerance phenotypes in another invertebrate model, *C. elegans*,[38] it is possible that, and testable whether, mutants affecting initial resistance also affect the development of acute tolerance, similar to the way they affect rapid tolerance.

A previous study has assessed sedation and tolerance phenotypes of *Gprk2* mutants.[22] These mutants were considerably more resistant and much less tolerant than control flies, consistent with the trend of our inverse correlation. Based on these findings, the authors of that study also wondered whether the *Gprk2* tolerance phenotype was a result of the initial resistance phenotype. To investigate this, they exposed *Gprk2* mutants and control flies to the same depth of sedation by exposing them to the ST-90, the time point at which 90% of the flies in each vial were sedated. After exposing flies to the ST-90, which took longer for *Gprk2* mutants, they still developed significantly less tolerance than controls and are, therefore, primary tolerance mutants. This is also suggested by their residual of −4.85 from our correlation with the initial same-dose exposure data (Figure 9C). Exposure of both the experimentals and controls to the ST-90 is conceptually similar to our *GNMT* experiments (Figure 8), where both groups were exposed to 1.5*ST-50, the result of both paradigms being that experimental and control flies are exposed to a consistent depth of sedation. These methods of determining *Gprk2*, but not *GNMT*, to be primary tolerance mutants suggest that tolerance mutants may be most easily identified via a mechanism that exposes experimental and control flies to the same behavioral effect, such as the inebriometer.[39,40] In the inebriometer, flies are exposed to alcohol until they become sedated and elute out the bottom of the device, meaning that each fly is exposed to an equivalent dose of alcohol which confers a consistent behavioral effect. In the absence of an inebriometer, a similar exposure could be performed by exposing flies to 1.5*ST50, as we have done here, or to the ST-90, as performed by Kang and colleagues.[22]

Together, these findings suggest that initial resistance to alcohol sedation is a behavioral set point that impacts the subsequent development of tolerance. Accordingly, many mutants that are considered tolerance mutants may, in reality, be resistance mutants that appear to have tolerance phenotypes only because of their resistance phenotypes. Increasing the duration of the initial exposure increases the amount of tolerance developed.[1,10] Therefore, accurate assessment of potential tolerance mutants requires correcting for initial resistance phenotypes. As mentioned above, a clear indication of a primary tolerance mutant is that compared to the control, the mutant has a tolerance phenotype but no initial resistance phenotype (as is the case for mutants *PQBP1*$^{2\text{-}10}$ and *sgg*$^{5\text{-}21}$, Figure 9A). However, when a mutant has both an initial resistance and tolerance phenotype, it is less clear whether it is a primary tolerance mutant. Therefore, approaches to correcting for initial resistance would include testing potential tolerance mutants in an inebriometer or exposing experimental and control flies to the same sedation depth with exposures of 1.5*ST-50 or to ST-90. However, as a preliminary step to performing these experiments, one can take advantage of the linear regression equation we have generated here (which is informed by 158 unique data points) to assess potential tolerance mutants. We determined our residuals using the linear regression equation ($y = -0.56\ x - 0.23$). Because we used effect sizes for this correlation, they should be comparable from one experiment to the next regardless of the exact numerical naïve resistance and tolerance values.

We, therefore, provide the following 'how-to' guide for using our linear regression equation to calculate residuals and assess potential tolerance mutants. This approach applies to experiments where experimental versus control flies are exposed to a set dose/duration of ethanol, with subsequent re-exposure to determine tolerance. First, determine the initial ST-50 and second (tolerance) ST-50 for mutant and control flies, as described previously.[12] Then, calculate the effect sizes[14] for resistance and tolerance (using, e.g., this tool, where Group 1 is the control flies, and Group 2 is the experimental flies: https://www.psychometrica.de/effect_size.html. We recommend using #2, Hedge's *g*, which controls for small sample sizes). Since the effect size is highly sensitive to the standard deviation, we recommend normalizing effect sizes if the standard deviation appears to be much lower or higher than is typical. Next, use our equation ($y = -0.56\ x - 0.23$), and plug in the initial resistance effect size for *x*. Then, subtract the result of this calculation from the effect size for tolerance measured experimentally. This will provide the residual for the mutant assayed.

As an example, we calculated the initial resistance and tolerance effect sizes for *GNMT*, *Arf6*, and *Gprk2* and plugged these into the

equation. After subtracting the calculated values from their tolerance effect sizes, we found residuals of 0.18, −3.91, and −4.85, respectively (Figure 9C,D). These residuals support the conclusions that *GNMT* is not a primary tolerance mutant, while *Arf6* and *Gprk2* are both primary tolerance mutants, as determined by our findings and as reported previously.[22] We suggest that utilizing our equation may be a useful starting point for determining promising candidate tolerance mutants to follow up on. Any mutants with absolute residuals from this equation around 2 or higher are most likely strong, primary tolerance mutants, and any with absolute residuals between 1.3 and 2 may be worth investigating more closely. Should these candidates show a naïve resistance phenotype, this might include adjusting the first exposure to the same effective dose, as we have done here with genotypes exposed to 1.5*ST-50, or as done by Kang et al.,[22] exposed to ST-90. Our data are thus widely applicable for understanding the mechanisms of alcohol-induced tolerance, an important but understudied endophenotype of AUD.[3]

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DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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