2014

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Published in final edited form as:
Alcohol Clin Exp Res. 2014 June ; 38(6): 1582–1593. doi:10.1111/acer.12421.

Contrasting influences of *Drosophila white/mini-white* on ethanol sensitivity in two different behavioral assays

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Abstract

Background—The fruit fly *Drosophila melanogaster* has been used extensively to investigate genetic mechanisms of ethanol-related behaviors. Many past studies in flies, including studies from our laboratory, have manipulated gene expression using transposons carrying the genetic-phenotypic marker *mini-white*, a derivative of the endogenous gene *white*. Whether the *mini-white* transgenic marker or the endogenous *white* gene influence behavioral responses to acute ethanol exposure in flies has not been systematically investigated.

Methods—We manipulated *mini-white* and *white* expression via (i) transposons marked with *mini-white*, (ii) RNAi against *mini-white* and *white* and (iii) a null allele of *white*. We assessed ethanol sensitivity and tolerance using a previously described eRING assay (based on climbing in the presence of ethanol) and an assay based on ethanol-induced sedation.

Results—In eRING assays, ethanol-induced impairment of climbing correlated inversely with expression of the *mini-white* marker from a series of transposon insertions. Additionally, flies harboring a null allele of *white* or flies with RNAi-mediated knockdown of *mini-white* were significantly more sensitive to ethanol in eRING assays than controls expressing endogenous *white* or the *mini-white* marker. In contrast, ethanol sensitivity and rapid tolerance measured in the ethanol sedation assay were not affected by decreased expression of *mini-white* or endogenous *white* in flies.

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Conclusions—Ethanol sensitivity measured in the eRING assay is noticeably influenced by *white* and *mini-white*, making eRING problematic for studies on ethanol-related behavior in *Drosophila* using transgenes marked with *mini-white*. In contrast, the ethanol sedation assay described here is a suitable behavioral paradigm for studies on ethanol sedation and rapid tolerance in *Drosophila* including those that use widely available transgenes marked with *mini-white*.

Keywords

alcohol; behavior; fruit flies; genetics; intoxication; sedation

Introduction

Although many studies have collectively suggested the involvement of a large number of genes in human alcohol use disorders (AUDs) (Palmer et al., 2012), few genes have been unambiguously associated with alcohol dependence or other aspects of alcohol abuse. The fruit fly *Drosophila melanogaster*—which exhibits behavioral responses to ethanol that mirror those observed in mammals (Devineni et al., 2011; Scholz et al., 2000)—has been used to identify candidate genes for subsequent studies on AUDs in humans and for directly testing the roles of genes implicated by human studies (Rodan and Rothenfluh, 2010; Scholz and Mustard, 2011). The advent of transposon-mediated mutagenesis and transgenesis (Rubin and Spradling, 1982) greatly facilitated the use of the fly model to investigate genes that influence many biological processes including behavioral responses to ethanol (Bellen et al., 2011; Bellen et al., 2004; Rodan and Rothenfluh, 2010). For example, studies using transgenic flies have demonstrated that signaling via cyclic AMP, monoamines, insulin, and neuropeptides impact ethanol-related behaviors (Bainton et al., 2000; Corl et al., 2005; Moore et al., 1998; Rodan et al., 2002; Scholz, 2005; Wen et al., 2005). Additionally, the fly model has been used to establish roles for multiple cytoskeletal regulators and cell adhesion molecules (Bhandari et al., 2009; Peru y Colon de Portugal et al., 2012; Rothenfluh et al., 2006; Sordella and Aelst, 2006), chloride intracellular channels (Bhandari et al., 2012) and many other proteins (Rodan and Rothenfluh, 2010; Scholz and Mustard, 2011) in sensitivity and tolerance to ethanol.

A common feature of the majority of the genetic studies on ethanol behavior in *Drosophila* is the use of transformation vectors that contain a version of the *white* (*w*) gene as a selectable phenotypic marker (*mini-w*). The *w* gene product is an ABC transporter subunit thought to heterodimerize with the products of the *brown* and *scarlet* genes to form a functional transporter (Ewart and Howells, 1998; Mackenzie et al., 1999). White protein localizes to the endosome in pigment cells where it cooperates with Brown and Scarlet proteins to mediate the intracellular transport of guanine and tryptophan metabolites (Anaka et al., 2008; Mackenzie et al., 2000). Wild-type flies have red eyes, whereas null mutations in *w* lead to a complete loss of eye pigmentation (i.e. white eyes).

The *mini-w* mini-gene in many currently used *Drosophila* transformation vectors originates from the *pW6* (Klemenz et al., 1987) and *pCaSpeR* (Pirrotta, 1988) P-element vectors. The *mini-w* cassette from *pCaSpeR* (w^{+mC}) consists of ~300 bp of upstream and ~600 bp of

downstream regulatory *w* sequence, with most of the first intron removed. In the *pW6* vector, the minimal *w* promoter is replaced with the *Hsp70* minimal promoter ($w^{+mW.hs}$). Transformation of *w* null mutants with these vectors, or their many derivatives, rescues eye pigmentation through expression of White protein (i.e. causes eyes to be red). The convenient nature of the *w* eye color phenotype has made *mini-w* a routinely used marker of transgenesis in *Drosophila*. For example, most Gal4 and UAS transgenes used in flies are marked with *mini-w* (e.g. (Brand and Perrimon, 1993; Dietzl et al., 2007) and (at the time of manuscript preparation) more than 40% of the ~30,000 P-element vector-containing strains in the Bloomington *Drosophila* Stock Center (http://flystocks.bio.indiana.edu/) were marked with a version of *mini-w*. Although other markers for transgenesis are used in *Drosophila* (e.g. *yellow* and GFP (Bellen et al., 2011)), the *mini-w* marker has been used in many—bordering on all—genetic studies in flies.

The *w* gene product is highly conserved among many insects and is structurally related to the human protein ABCG1 that is associated with multiple mental health disorders (Kirov et al., 2001; Nakamura et al., 1999). In addition to the *Drosophila* eye, the *w* gene is highly expressed in the head outside of the eye (Campbell and Nash, 2001), in the prepupal fat body (functionally analogous to the vertebrate liver), and the adult Malpighian tubules (functionally analogous to the vertebrate kidney) (Chintapalli et al., 2007). The *w* locus could therefore be important for the function of the brain and other organ systems in addition to the eye in *Drosophila*. Consistent with this possibility, several studies have shown that endogenous *w* or *mini-w* influence multiple non-visual processes. Null mutants for *w* have altered levels and localization of the biogenic amines dopamine, serotonin, and histamine in heads (Borycz et al., 2008; Sitaraman et al., 2008), are resistant to sedation by volatile anesthetic gases (Campbell and Nash, 2001) and have poor place memory (Diegelmann et al., 2006). Expression of *w* is found in fly heads from which eye pigment cells have been genetically ablated (Campbell and Nash, 2001), supporting a role for *w* in anesthetic sensitivity and possibly other behaviors that are independent of vision. Additionally, *mini-w* over-expression induces male-male courtship behavior in flies (Anaka et al., 2008; Hing and Carlson, 1996; Nilsson et al., 2000; Zhang and Odenwald, 1995) and ethanol-induced male-male courtship behavior requires expression of *w* or *mini-w* (Lee et al., 2008). Expression of *w* and *mini-w* can therefore have significant effects on neurochemistry and behavior in *Drosophila*, possibly via a role in transport of guanine or cGMP and synthesis of nitric oxide, dopamine, serotonin or histamine (Borycz et al., 2008; Campbell and Nash, 2001; Evans et al., 2008; Sitaraman et al., 2008).

Given the use of the fly as a model for ethanol-related behaviors and the wide-spread use of *mini-w* as a transgenic marker in flies, we explored the contribution of *mini-w* and endogenous *w* expression to ethanol sensitivity in two different behavioral assays. We find that ethanol sensitivity measured by loss of climbing in flies is significantly influenced by *mini-w* and endogenous *w*, whereas ethanol sensitivity measured by sedation is not.

Materials and methods (additional details are provided as supplementary information on line)

Fly husbandry

Flies were grown on food medium (10% sucrose, 3.3% cornmeal, 2% yeast, 1% agar) supplemented with active dry yeast, 0.2% Tegosept (Sigma Chemical Co., St. Louis, MO, USA) and antibiotics (0.5 μg ampicillin, 0.1 μg tetracycline, 0.625 μg chloramphenicol per 10 ml of food) at 25°C/60% relative humidity with a 12 hour light/dark cycle. The w^{1118} control strain isogenic for the *X*, *2* and *3* chromosomes used in these studies (a.k.a. *w[A]*) was obtained from the *Drosophila* Stock Center (stock# 5905, Bloomington, IN, U.S.A.). The $elav^{C155}$*-Gal4* driver (*elav-Gal4*), *Clic* (*G4072* and *EY04209*), *Akap200* (*EP2254, c01373, d01782, d03938, d07255, EY04645* and *EY12242*), *thickveins* (*7, 8, d07811, f02766, f03305, c06013* and *KG05071*), *wishful thinking* (*d02492, e00566* and *e01243*) and *baboon* (*c04263, c05710, k16912*) strains were obtained from the Bloomington *Drosophila* Stock Center (Bloomington, IN, U.S.A.) or the Exelixis Collection at the Harvard Medical School (Boston, MA, U.S.A.) and backcrossed for 7 generations to the *w[A]* control to normalize their genetic background. An additional w^{1118} genetic background strain (*w[VDRC]*) and UAS-RNAi transgenic strains to manipulate *white* (*v30033* and *v30034*), *Clic* (*v105975*), *Cnx14D* (*v5597*) and *ph-p* (*v50024*) were obtained from the Vienna *Drosophila* RNAi Center (Vienna, Austria). An *X* chromosome harboring both the *elav-Gal4* driver and the *v30034* RNAi transgene (*elav-Gal4,v30034*) was generated via meiotic recombination. A stock homozygous for a wild-type *w* allele (w^+) in the *w[A]* background was generated by backcrossing a Canton-S *X* chromosome to *w[A]* for 7 generations. Unless otherwise indicated, all strains contained a w^{1118} *X* chromosome and all transgenic lines contained *mini-w*. The previously described scb^{Vol2}, $Alk^{MB06458}$, $aru^{8.128}$, $hppy^{KG5537}$, NPFR1-Gal4 and *NPFR1-RNAi* strains (Bhandari et al., 2009; Corl et al., 2009; Eddison et al., 2011; Lasek et al., 2011; Wen et al., 2005) and control genetic backgrounds were kindly provided by Ron Davis, Ulrike Heberlein and Ping Shen.

For behavioral analyses, adult flies (2–5 days-old) were grown as above, immobilized under light CO_2 anesthesia, separated by sex and genotype, and placed into fresh food vials (25 flies/vial for eRING, 11 flies/vial for sedation) overnight at 25°C/60% relative humidity. All comparisons between groups were based on flies that were grown, handled and tested side-by-side. Each vial of flies represents n=1. All behavioral assays were performed at 23–25°C and 50–55% relative humidity under standard laboratory lighting. The experimenter was blind to genotype in all studies.

eRING assay for ethanol-related behavior

eRING studies (Figure S1) were performed as previously described (Bhandari et al., 2009) using vapor from 33 or 50% ethanol. Flies have a strong, innate negative geotaxis response (vertical climbing in response to being startled). In eRING studies (Figure S1), bang-induced climbing distance in the continuous presence of ethanol vapor is measured at 1 min intervals. Flies become progressively sedated and T50 values (time to ethanol-induced 50% reductions in climbing) are determined as previously described (Bhandari et al., 2009).

Ethanol sedation assay for ethanol sensitivity and tolerance

Ethanol sedation assays (Figure S1 and S3) were initiated by transferring adult flies into empty 2.5 × 9.5 cm food vials (VWR; Radnor, PA, U.S.A.; catalogue number 89092-722).

Cellulose acetate Flugs (FlyStuff.com; San Diego, CA, U.S.A.; catalogue number 49-102) were inserted 2 cm into each vial and the vials of flies were arranged into six rows of four vials each (Figure S3). The number of dead/inactive flies (on average <1%) was recorded for each vial at t=0 min and censored. Starting with the first row of 4 vials, ethanol (0–50%; 2 ml in our standard assay) was added at five-second intervals to the Flug in each vial and the vial was immediately sealed with a silicone stopper. The remaining rows of vials were treated identically at 1-minute intervals. Starting 6 minutes after adding ethanol to the Flugs and continuing at 6-minute intervals thereafter, each vial was gently tapped 3 times on a table and the number of sedated flies (i.e. flies that were noticeably uncoordinated or immobile) in each vial was recorded 30 seconds after the final tap. Ethanol sedation assays were terminated typically at 60–90 minutes or when all flies were immobile. The percentage of non-sedated flies was calculated for each vial at each 6-minute interval, resulting in a sedation time-course for each vial. Sedation time 50 (ST50, time to 50% sedation) values were interpolated from third-order polynomial curve fits (the least complex curve that fit the data well, (R^2 = 0.96±0.001, n=1221) using Excel (Microsoft, Redwood, WA, U.S.A.) or Prism 4.03 (GraphPad, San Diego, CA, U.S.A.) from the time-course data for each vial.

Rapid tolerance was determined in ethanol sedation assays as described in the preceding paragraph except that flies were given a first exposure to ethanol (E), allowed to recover for 4 hours in food vials at 25°C/60% relative humidity, and then subjected to a second ethanol exposure (EE) in ethanol sedation assays. The development of rapid tolerance to ethanol was expressed as a ratio between the $ST50_{EE}$ and $ST50_{E}$ as similarly reported (e.g.(Awofala et al., 2011; Berger et al., 2004; Scholz et al., 2005; Scholz et al., 2000)).

Results and Discussion

eRING assays and expression of mini-w and w

Our laboratory previously described eRING (ethanol rapid iterative negative geotaxis) as an assay for measuring ethanol sensitivity in *Drosophila* (Figure S1) (Bhandari et al., 2009). Flies have a strong, innate negative geotaxis response (vertical climbing in response to being banged or rapped to the bottom of their container). eRING assays measure bang-induced climbing at one-minute intervals in the continuous presence of ethanol vapor. Flies are banged to the bottom of vials during each interval in eRING assays, right themselves and then climb toward the top of the vials. As the internal ethanol concentration of flies increases in eRING assays, flies become progressively impaired which is reflected as a time-dependent decrease in the distance climbed. Time-course data from eRING assays are used to derive a T50 (time of ethanol exposure causing a 50% reduction in climbing ability) as a standard measure of fly performance and ethanol sensitivity.

While performing a reverse genetic screen with transposon insertion strains, we noticed that genotypes with increased resistance to ethanol sedation in eRING assays also often had strongly pigmented eyes from the *mini-w* eye color marker in the transposons. We therefore investigated the potential confound of *mini-w* expression in eRING studies by assessing ethanol sensitivity in three series of fly strains with graded levels of *mini-w* expression from transposon insertions (Figure S2). Expression of *mini-w*, which varies greatly in different transposon strains due to well-documented position effects (Hazelrigg et al., 1984; Silicheva

et al., 2010), was ranked in our studies by eye color (w^+-rank) by a single experimenter blind to genotype. The series of flies we assessed in eRING harbored (i) transposon insertions in three TGFβ receptor (TGFβR) genes (*thickveins* (*tkv*), *wishful thinking* (*wit*), and *baboon* (*babo*)), (ii) transposon insertions in the *Akap200* locus, and (iii) several different Gal4 transgenes, all marked with *mini-w* in the same w^{1118} genetic background. Expression of *mini-w* from these transposon insertions strongly correlated with ethanol sensitivity in eRING assays (Figure 1A), but not with internal ethanol concentration, expression of TGFβR or *Akap200* mRNA, or negative geotaxis in the absence of ethanol (not shown). These results suggest that expression of *mini-w* influences sensitivity to ethanol in the eRING paradigm.

To formally investigate *mini-w* in ethanol sensitivity in eRING assays, we used nervous system Gal4 (*elav-Gal4*) (Olofsson and Page, 2005) to drive expression of a *UAS-white-RNAi* transgene (*v30034*) to knockdown *mini-w* (Figure S2I, K and M). In all experiments, the *elav-Gal4* and *UAS-white-RNAi* transposons contained the *mini-w* marker (Figure S2). White-eyed flies with the w^{1118} null allele or with RNAi-mediated knockdown of *mini-w* (*elav-Gal4,v30034*) were significantly more sensitive to ethanol in eRING assays than red-eyed flies expressing *mini-w* from the *elav-Gal4* (*elav-Gal4*/+) or *v30034* (*v30034*/+) transgenes (Figure 1B). Additionally, w^{1118} null flies were much more sensitive to ethanol in eRING studies than flies with a wild-type allele of *w* (w^+) in the same genetic background (Figure 1C). Both *mini-w* and endogenous *w*, therefore, have substantial effects on ethanol sensitivity in the eRING assay. Given the widespread use of flies with altered expression of *w* (for example, >75% of the ~46,000 stocks in the *Drosophila* Bloomington Stock Center contain an allele of *w* or *mini-w* at the time of manuscript preparation), our findings represent a significant limitation to the utility of the eRING assay for the genetic analysis of ethanol sensitivity in *Drosophila.* A more detailed analysis that addresses possible mechanisms for the effect of *mini-w* and endogenous *w* on ethanol sensitivity in eRING studies will be presented elsewhere.

Ethanol sensitivity and rapid tolerance in an assay based on sedation

We investigated a behavioral paradigm based on ethanol-induced sedation to potentially circumvent the confound of *mini-w* and *w* in eRING assays. The ethanol sedation assay we developed is based in large part on the work of others (e.g. Lasek et al., 2011; Maples and Rothenfluh, 2011; Rothenfluh et al., 2006; Schumann et al., 2011; Wen et al., 2005). In the ethanol sedation assays used in our studies, flies were placed in a 9.5 cm tall plastic food vial and trapped in the vial with a cellulose acetate Flug (Figure S3A). Ethanol solution (up to 2 ml, Figure S4A) was added to the top (exposed side) of the Flug and the vial was immediately sealed with a rubber stopper (Figure S3A). At 6-minute intervals thereafter, flies were gently tapped to the bottom of the vial and then visually scored for their ability to right themselves (i.e. stand up) in the continuous presence of ethanol vapor from the Flug. We designed our ethanol sedation assay to test multiple replicates of several genotypes in parallel (Figure S3B). Each vial of flies corresponded to n=1 and we found that an individual experimenter can readily test 24 vials simultaneously in a single experiment using this design. Testing more than 24 vials simultaneously certainly seemed possible assuming minor modifications, but we have not pursued this possibility.

The primary data from ethanol sedation assays are the percentages of non-sedated flies measured as a function of ethanol exposure time (e.g. Figure 2A and 2B). The time required for 50% of flies to become sedated (Sedation Time 50, ST50) is a metric routinely extracted from similar ethanol sedation time-course studies (e.g. (Schumann et al., 2011)). Toward having a uniform, objective strategy for data analysis, we interpolated ST50 values from curve fits of our ethanol sedation time-courses. Third-order polynomials fit ethanol sedation time-course data well (R^2 = 0.96±0.001, n=1221) and third-order polynomial curves fit the ethanol sedation time-course data better than first-, second- or fourth-order curves (Figure S4B). Additionally, we found that ST50s and the percentage of active flies integrated over time (area under the curve) from ethanol sedation time-course data sets strongly correlated (Figure S4C), indicating that the ST50 metric captures the overall performance of flies in this assay. We therefore used ST50 values interpolated from third-order polynomial curves as end measures of ethanol sensitivity in all ethanol sedation studies described here. Note that lower and higher ST50s indicate increased and decreased ethanol sensitivity, respectively.

To determine if flies were sensitive to ethanol dose in ethanol sedation assays, we tested control w^{1118} flies in the presence of water vapor or vapor from 30–50% ethanol. Neither females nor males became sedated in the presence of water vapor (Figure 2A, 2B, 3A, 3B and S5A). In contrast, exposure to vapor from increasing concentrations of ethanol progressively hastened time-dependent sedation (females, Figure 2A; males, 2B) and therefore also decreased ST50s (females, Figure 2C; males, 2D). Exposure to vapor from increasing concentrations of ethanol also increased the internal ethanol content of flies (females, Figure 2E; males, 2F), demonstrating that sedation in the ethanol sedation assay is dose-dependent. Although in some of our initial studies we noticed that ST50s in w^{1118} females and males appeared to be different when tested on different days in separate experiments (e.g. Figure 2C and 2D), we found that males and females performed indistinguishably when tested on the same day side-by-side (Figure S5A and S5B). Therefore, comparisons were made only between groups tested on the same day. Flies in ethanol sedation assays lost a comparable amount of body mass when exposed to vapor from water or ethanol (Figure S5C), indicating that sedation in the presence of ethanol vapor is not due to dehydration.

Rapid tolerance is defined as a reduction in ethanol sensitivity during a second exposure to the drug following recovery from an earlier initial exposure (Devineni et al., 2011; Scholz, 2009). Control w^{1118} flies became sedated during both a first (E) and second (EE) ethanol exposure separated by four hours of recovery in ethanol sedation assays, but they were significantly less sensitive during the second challenge with ethanol (females, Figure 3A and C; males, Figure 3B and D). Ethanol sensitivity following an initial exposure to water (WE group) was not altered compared to flies with no prior ethanol experience (E group; Figure 3A–D), indicating that blunted ethanol sensitivity in EE flies is not due to a handling artifact and instead requires multiple exposures to the drug. Internal ethanol concentrations were indistinguishable during a first and second ethanol exposure (females, Figure 3E; males, Figure 3F). We conclude that the decreased ethanol sensitivity during the second ethanol exposure in ethanol sedation assays is due to altered pharmacodynamic properties of the

drug and that this change in behavior represents the development of functional rapid tolerance. To date, we have been unable to show that flies develop acute functional tolerance (tolerance during a single ethanol exposure (Davies et al., 2004)) in ethanol sedation assays (Figure S6).

Mini-w, ethanol sensitivity and rapid tolerance in ethanol sedation assays

We used the ethanol sedation assay to address the potential influence of *mini-w* expression on ethanol sensitivity and rapid tolerance. We first assessed initial ethanol sensitivity in *Akap200* and TGFβR transposon insertion genotypes in addition to strains containing several different Gal4 transgenes, all marked with *mini-w* in the same genetic background. There was no correlation between ST50s from ethanol sedation assays and expression of *mini-w* in these studies (Figure 4A). These results suggest that *mini-w* does not influence ethanol sensitivity measured in ethanol sedation experiments, in contrast to our data from eRING assays (Figure 1).

To further investigate the potential role of *mini-w* in ethanol sedation assays, we determined if RNAi-mediated knockdown of *mini-w* in the nervous system via *elav-Gal4* (Olofsson and Page, 2005) altered ethanol sensitivity in this paradigm. In all experiments, the *elav-Gal4* and *UAS-white-RNAi* transposons themselves contained the *mini-w* marker (Figure S2). Nervous system expression of two *w* RNAi transgenes (*v30033* and *v30034*) led to eye color phenotypes indistinguishable from w^{1118} null flies (Figure S2I–M), demonstrating that expression of *v30033* and *v30034* substantially inactivates *mini-w*. ST50s from ethanol sedation assays were not significantly different in w^{1118} nulls and red-eyed control flies expressing *mini-w* (*elav-Gal4/+*, *v30033/+* and *v30034/+*; Figure 4B). Ethanol sensitivity in white-eyed flies expressing *w* RNAi in the nervous system (*elav-Gal4;v30033* and *elav-Gal4/v30034*) was significantly increased compared to the *elav-Gal4/+* control, but not compared to the *v30033/+* or *v30034/+* controls (Figure 4B), indicating that knockdown of mini-w has a negligible effect. Internal ethanol concentrations were comparable in all control and *w* knockdown strains tested (Figure 4C). Additionally, the development of rapid tolerance was observed in all control and *w* knockdown groups (Figure S7). Although rapid tolerance was slightly lessened in *elav-Gal4/v30034 w*-knockdown flies compared to *elav-Gal4/+* and *v30034/+* controls, this decrease in rapid tolerance was not found in *elav-Gal4;v30033* knockdown flies (Figure S7). We conclude that—in genetic backgrounds with essentially normal ethanol sensitivity—neither expression of *mini-w* from stably-integrated transposons nor knockdown of *mini-w* in the nervous system greatly alters ethanol sedation sensitivity, rapid tolerance to ethanol or ethanol uptake/metabolism in flies as measured in ethanol sedation assays.

The preceding data strongly indicate that *mini-w* does not impact behavioral performance in ethanol sedation assays using flies with essentially normal ethanol sensitivity. We reasoned, however, that *mini-w* could have subtle effects on performance in ethanol sedation assays that would be revealed in flies with altered baseline ethanol sensitivity. To test this possibility, we co-expressed *w* RNAi in conjunction with UAS-RNAi transgenes against *Cnx14D* (*v5597*) or *ph-p* (*v50024*). Expression of the *Cnx14D* and *ph-p* UAS-RNAi transgenes (identified in a reverse genetic screen that will be reported elsewhere) decrease

and increase ethanol sensitivity, respectively (Figure 5A and 5B). To achieve coincident expression of RNAi against *w* and either *Cnx14D* or *ph-p*, we generated flies containing a recombinant *X* chromosome harboring an *elav-Gal4* driver and the *v30034* RNAi transgene in *cis*. Eye pigmentation in flies with this recombinant chromosome (*elav-Gal4,v30034*) was indistinguishable from w^{1118} null flies (Figure S2A), indicating strong knockdown of *mini-w*. Ethanol sensitivity in ethanol sedation assays was statistically indistinguishable in w^{1118} nulls, flies expressing *mini-w* (*elav-Gal4/+* and *v5597/+* controls) and *w* knockdown flies (*elav-Gal4,v30034/+*) (Figure 5A). Expression of *Cnx14D* RNAi *v5597* with *elav-Gal4* (*elav-Gal4/v5597*) led to the expected increase in ST50, but importantly this phenotype was not significantly affected by coincident knockdown of *mini-w* (*elav-Gal4,v30034/v5597*) (Figure 5A). Similarly, we found that ethanol sensitivity in w^{1118}, *elav-Gal4,v30034/+*, and *elav-Gal4/+* controls were comparable and that the increased sensitivity of flies expressing *ph-p* RNAi *v50024* was not affected by concurrent knockdown of *mini-w* (Figure 5B). Although additional studies will be required to determine if knock-down of Cnx*14D* and *ph-p* alter ethanol sensitivity in flies, these data show that *mini-w* has no significant effect on the phenotypes of flies with increased or decreased sensitivities to ethanol.

Endogenous w and ethanol sedation assays

We next examined the effects of endogenous *w* on sensitivity and rapid tolerance to ethanol in ethanol sedation assays. We found no differences in initial ethanol sensitivity in w^{1118} null and *w* wild-type (w^{+}) females or males tested with vapor from 50% (Figure 6A and B) or 40% ethanol (Figure S8A). The development of rapid tolerance to ethanol was similarly unaffected by *w* genotype in either sex (Figure S8B and C). Additionally, there were no significant differences in internal ethanol concentrations (Figure 6C and 6D) in w^{1118} and w^{+} flies. These results indicate that endogenous *w*, like *mini-w*, has no discernible effect on ethanol sensitivity, rapid ethanol tolerance or ethanol kinetics in ethanol sedation assays.

Chloride intracellular channels and other molecules influence ethanol sedation assays

We previously reported that genes in the *Chloride Intracellular Channel* (*CLIC*) family influence ethanol sensitivity in flies, worms and mice (Bhandari et al., 2012). In the previous *Drosophila* experiments, we used eRING assays to measure ethanol sensitivity in flies harboring two independent *Clic* transposon insertions marked with *mini-w*. Given the data in Figure 1, it seemed possible that the decreased ethanol sensitivity exhibited by *Clic* mutants —as measured in eRING assays—could be confounded by the presence of the *mini-w* marker. We therefore used ethanol sedation assays to re-examine ethanol sensitivity in the same two *Clic* transposon mutants. Consistent with our previous eRING studies, we found that both *Clic* mutants had decreased ethanol sensitivity in ethanol sedation assays (Figure 7A and B). Similarly, a 40±4% knockdown (one-sample t test, p=0.0007, n=5) of *Clic* via ubiquitous *da-Gal4*-driven expression of the *Clic* RNAi transgene *v105975* also lessened ethanol sensitivity in sedation assays without having a major effect on internal ethanol concentrations (Figure 7C and E). Nervous system expression of *Clic* RNAi *v105975* also decreased ethanol sensitivity without substantively impacting internal ethanol concentrations (Figure 7D and F). Therefore, decreased function of *Clic* alters ethanol sensitivity measured in sedation experiments. Importantly, these behavioral changes are independent of *mini-w*

because ethanol sensitivity is not greatly influenced by *mini-w* or endogenous *w* in sedation assays (Figures 4–6).

To determine if the ethanol sedation assay described here was influenced by the same genetic manipulations that alter ethanol sensitivity in other studies, we analyzed the behavior of several previously described mutants with altered ethanol sensitivity. Ethanol sensitivity in our sedation assay was increased in *scb* and *aru* mutants, decreased in *Alk* and *hppy* mutants, and decreased in flies with knockdown of *NPF* (Figure S9), consistent with previous reports (Bhandari et al., 2009; Corl et al., 2009; Eddison et al., 2011; Lasek et al., 2011; Wen et al., 2005). Sensitivity to ethanol sedation in the assay described here seems to be influenced by the same or similar mechanisms as reported for other behavioral paradigms.

Summary

We tested the influence of *w* and *mini-w* on ethanol sedation in two behavioral assays, one based on bang-induced climbing (eRING) and another based on ethanol sedation. Knock-down of *mini-w* and a null mutation in the endogenous *w* locus increased sensitivity to ethanol sedation in eRING assays, whereas the same genetic manipulations of *mini-w* and *w* had no major effect on ethanol sensitivity measured in sedation assays. Therefore, the eRING assay should not be used for measuring ethanol sensitivity in studies with transposons marked with *mini-w* or studies that otherwise compare genotypes with unequal expression of *w* or *mini-w*. Considering the wide-spread use of transposons marked with *mini-w* and that expression of *mini-w* can vary considerably between independent transposon insertion strains (Hazelrigg et al., 1984; Silicheva et al., 2010), this is a significant limitation to the utility of the eRING assay for genetic studies on ethanol sensitivity in *Drosophila*. In contrast, the ethanol sedation assay described here can be used as an experimental platform for probing the genetic basis for ethanol sensitivity and tolerance using many existing fly strains including those with *mini-w* transgenes.

Although eRING and ethanol sedation assays both measure impaired motor/postural function in the continuous presence of ethanol vapor (Figure S1) and similar internal ethanol concentrations are required to cause impairment in both assays (100–150 mM; Figure 2E, 2F, 3E, 3F, and 4C for ethanol sedation and (Bhandari et al., 2012; Bhandari et al., 2009) for eRING), there are fundamental operational differences between the two behavioral paradigms (Figure S1) that could explain the strikingly different effects of *w/mini-white*. In eRING assays, flies are sharply banged to the bottom of their container, must right themselves and then climb. In ethanol sedation assays, flies are gently tapped to the bottom of their container, must right themselves and then walk. Thus, although behavioral performance in both assays is dependent on the righting reflex, eRING and sedation assays ultimately measure ethanol-induced impairment in climbing and walking, respectively.

Our studies suggest that *w* and *mini-w* specifically impact climbing in the presence of ethanol. One possible explanation for the contrasting effects of *w* and *mini-w* on climbing versus walking could be that these two behaviors are driven by different sets of neurons and that *w* and *mini-w* selectively affect the function of the neurons involved in climbing. The selective role of *w* and *mini-w* in climbing neurons could be related to cell-specific

expression of the genes *brown* and *scarlet* (known partners for the white transporter (Ewart et al., 1994)), neuron-specific release of or responses to nitric oxide, dopamine, serotonin or histamine (neuromodulators known or suspected to be altered in *w* mutants (Borycz et al., 2008; Campbell and Nash, 2001; Sitaraman et al., 2008)), or transport of cGMP (Evans et al., 2008). Alternatively, it is possible that climbing is simply more physically demanding than walking; if loss of *w* causes partial defects in neuronal function, those defects might be revealed during climbing in the presence of ethanol in eRING assays. Additional studies are needed to address these and other possible mechanisms for the effect of *w* and *mini-w* in ethanol sensitivity measured in eRING assays.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

Sources of Support: These studies were supported by the National Institute on Alcoholism and Alcohol Abuse (NIH) via the grants P20AA017828 (J.B. and M.G.), R01AA020634 (M.G.) and R01AA016837 (J.B.).

The authors thank Lauren Thomas for expert technical assistance and Kenneth Kendler, Michael Miles, Andrew Davies, Brien Riley and Danielle Dick of the Virginia Commonwealth University Alcohol Research Center for helpful discussions.

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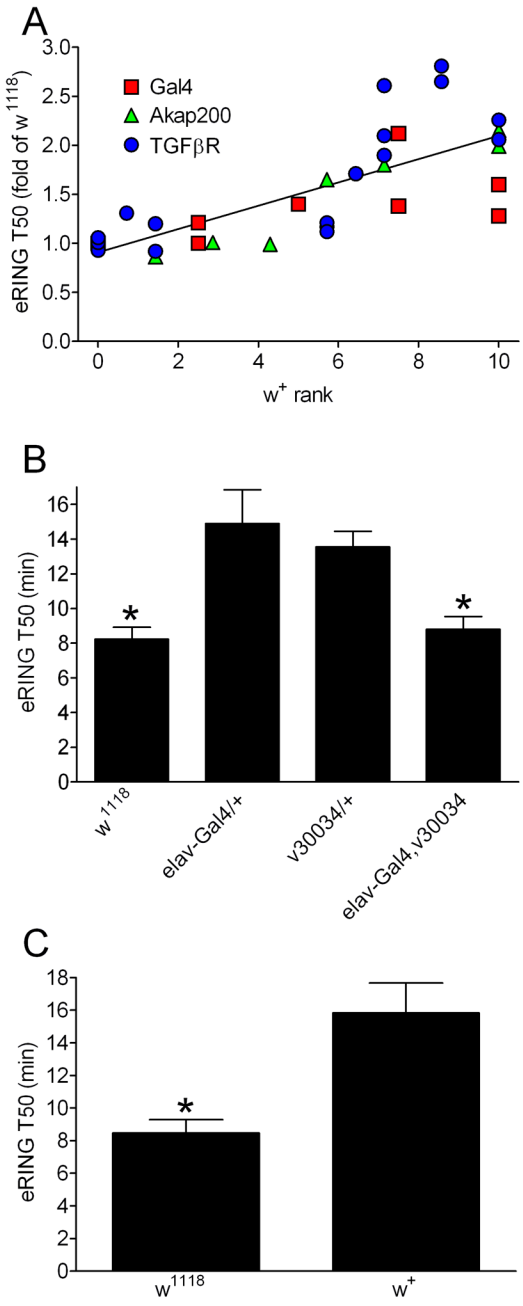

Figure 1. *Mini-w* and endogenous *w* influence ethanol sensitivity measured in eRING assays

A. Flies harboring transposon insertions in TGFβR genes (*tkv*, *wit* and *babo*; blue circles), *Akap200* (green triangles) or Gal4 drivers (red squares) were ranked by eye color (w^+ rank, X axis) and tested in eRING assays for sedation to ethanol vapor from 30% ethanol (TGFβR and Gal4 drivers) or 50% ethanol (*Akap200*). Compiled T50 values (fold of w^{1118} controls) from all genotypes correlated with w^+ rank (Pearson r=0.7503, p<0.0001). TGFβR lines tested were *tkv* alleles *7, 8, d07811, f02766, f03305, c06013* and *KG05071*, *wit* alleles *d02492, e00566* and *e01243* and *babo* alleles *c04263, c05710, k16912*. *Akap200* lines tested were *EP2254, c01373, d01782, d03938, d07255, EY04645* and *EY12242*. Gal4 lines tested

were *da-Gal4/+*, *mef2-Gal4/+*, *Appl-Gal4/+*, *Actin5cGal4/+*, *GMR-Gal4/+*, *24B-Gal4/+*, *and elav-Gal4/+*. See Figure S3 for representative eye color images. B. There was an overall effect of genotype on T50s from eRING studies using vapor from 30% ethanol (one-way ANOVA, p=0.0003, n=10 per genotype). *mini-w*-expressing *elav-Gal4/+* and *v30034/+* flies had elevated T50 values compared to white-eyed w^{1118} controls and *elav-Gal4,v30034 white* knockdown flies (*Bonferroni's, $p<0.05$). T50s in w^{1118} controls and *elav-Gal4,v30034* flies were not distinguishable (Bonferroni's, n.s.). C. T50s in w^{1118} flies tested in eRING studies with vapor from 30% ethanol were significantly lower than in w^{+} flies (Kolmogorov-Smirnov test for Gaussian distribution; w^{1118}, $p>0.01$, n.s.; w^{+}, p=0.0017, significantly non-Gaussian; *Mann-Whitney test, p=0.0147, n=10).

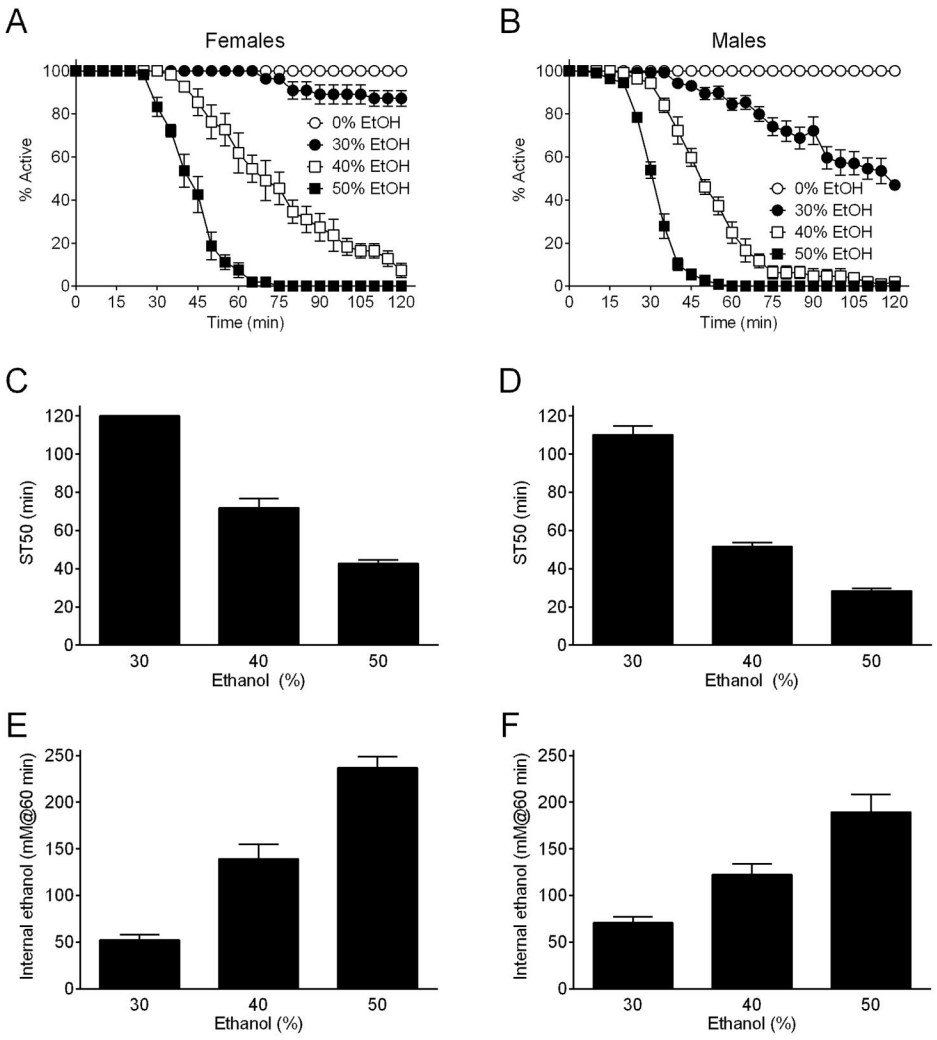

Figure 2. Exposure to ethanol vapor in ethanol sedation assays causes dose-dependent sedation and internal ethanol concentrations

Data are from w^{1118} control female (A, C and E) and male (B, D and F) flies exposed to vapor from the indicated concentrations of ethanol (0, 30, 40 and 50%). A and B. Ethanol sedation time-course. Time and ethanol concentration had significant effects on percent active flies and there was a significant interaction between time and ethanol concentration for both females and males (individual two-way ANOVAs; time, $p<0.0001$; ethanol concentration, $p<0.0001$; interaction, $p<0.0001$; n=5 for females, n=10 for males). C and D. Ethanol sedation ST50 values. ST50 values derived from the data in panels A and B were significantly affected by ethanol concentration in both males and females (individual one-way ANOVAs, $p<0.0001$, n=5 for females, n=10 for males). ST50 values in response to all ethanol concentrations were significantly different (Bonferroni's multiple comparison, $p<0.001$ in all cases). ST50 values cannot be calculated for flies exposed to 0% ethanol (water) because flies do not become sedated in the absence of the drug. E and F. Internal ethanol concentrations. A 60-minute exposure to vapor from increasing concentrations of ethanol progressively increased whole body internal ethanol concentrations in flies (individual one-way ANOVAs, $p\leq0.0002$, n=6 for females, n=5 for males). Internal ethanol

after any given exposure was significantly different from internal ethanol in the next lower and higher groups (Bonferroni's, $p<0.05$).

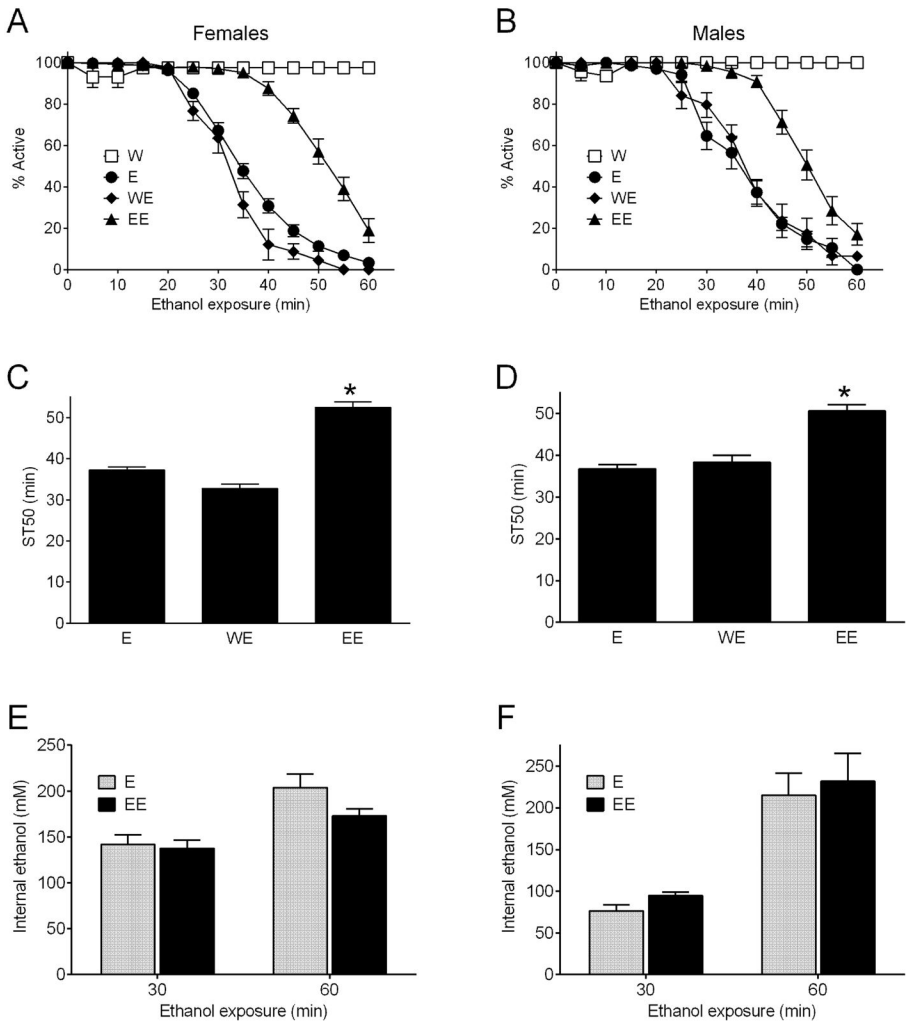

Figure 3. Rapid tolerance to ethanol in ethanol sedation assays

Data are from w^{1118} control female (A, C and E) and male (B, D and F) flies. A and B. Sedation time-courses from flies exposed once to vapor from water (W), exposed once to vapor from 50% ethanol (E), exposed to water vapor, allowed to recover for 4 hours, then exposed to vapor from 50% ethanol (WE), and exposed to vapor from 50% ethanol, allowed to recover for 4 hours, then exposed again to ethanol vapor (EE). Time and ethanol treatment had significant effects on the percentage of active flies and there was an interaction between time and ethanol treatment (individual two-way ANOVAs; time, $p<0.0001$; ethanol treatment, $p<0.0001$; interaction, $p<0.0001$, n=5–32 per treatment group). C and D. ST50 values derived from the data in panels A and B were significantly affected by ethanol treatment (one-way ANOVA, $p<0.0001$). ST50 values in EE flies were significantly different from those in E and WE flies (*Bonferroni's, $p<0.001$), whereas ST50 values in E and WE flies were not statistically distinguishable (Bonferroni's multiple comparison, n.s.). E and F. Internal ethanol concentrations increased with time of ethanol exposure, but were not significantly different in E and EE flies (individual two-way ANOVAs; time, $p\leq0.0002$; E vs. EE, n.s.).

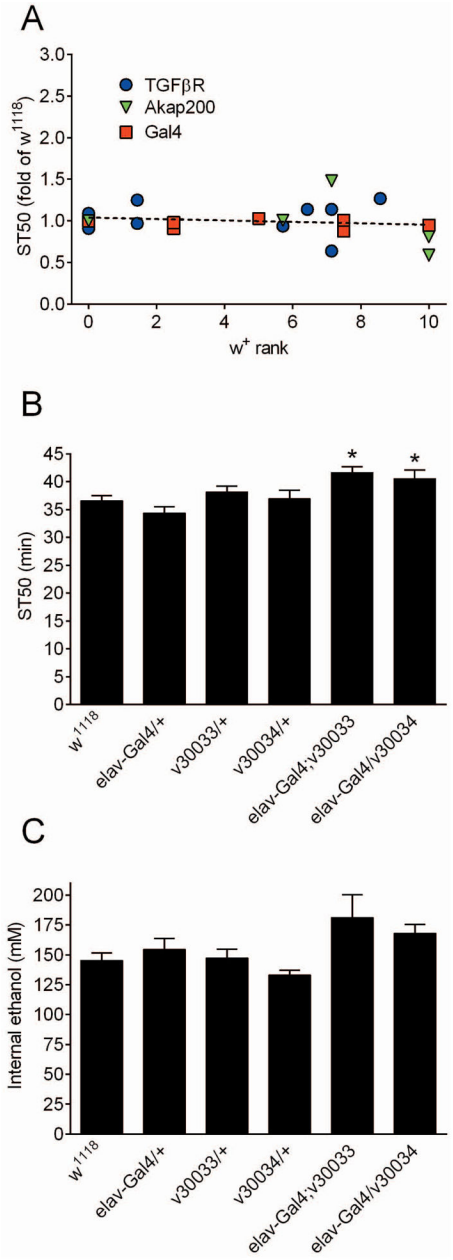

Figure 4. Expression of *mini-w* has a negligible impact on ethanol sedation sensitivity and internal ethanol concentrations in ethanol sedation assays

A. Compiled ST50 values from ethanol sedation assays with vapor from 50% ethanol did not correlate with w^+ rank in TGFβR (blue circles), *Akap200* (green triangles) and Gal4 (red squares) strains (Pearson r=−0.1754, p=0.4125, n.s.). ST50 values are represented as fold of w^{1118} controls. B. Knockdown of *mini-w* in the nervous system and initial sensitivity to ethanol. Expression of *w* RNAi transgenes (*v30033* and *v30034*) was driven in the nervous system by *elav-Gal4*. Genotype had a significant overall effect on ST50 values from ethanol sedation assays with vapor from 50% ethanol (one-way ANOVA, p=0.0008, n=8–16 per genotype). ST50 values in w^{1118}, *elav-Ga4/+*, *v30033/+* and *v30034/+* genotypes were not statistically different (Bonferroni's, n.s.). ST50 values in *elav-Gal4;v30033* and *elav-Gal4/*

v30034 knockdown animals were greater than in *elav-Ga4/+* (Bonferroni's, *p<0.05), but were not significantly different from *v30033/+* or *v30034/+* controls (Bonferroni's, n.s.). C. Internal ethanol concentrations in nervous system *mini-w* knockdown flies after 30 minutes of exposure to vapor from 50% ethanol in ethanol sedation assays. Genotype had a significant overall effect on internal ethanol (one-way ANOVA; p=0.0388; n=4), but no differences between relevant genotype pairs were found (Bonferroni's, n.s.).

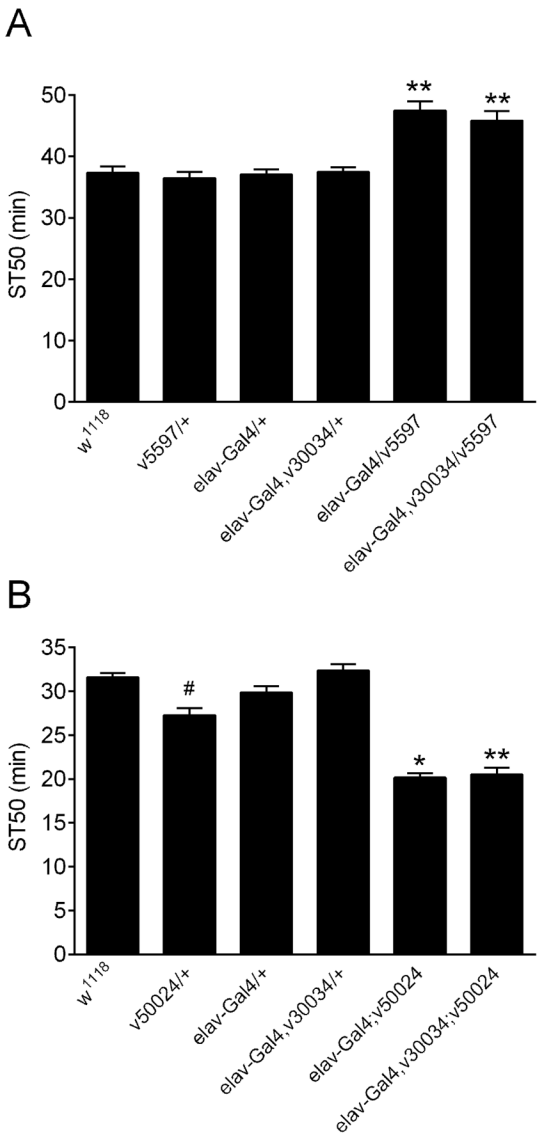

Figure 5. Nervous system knockdown of *mini*-w in flies with altered sensitivity to ethanol

Expression of *v30034* along with either *Cnx14D* RNAi *v5597* (A) or *ph-p* RNAi *v50024* (B) RNAi was driven in the nervous system by *elav-Gal4*. All flies tested were females. A. Knockdown of *mini-w* in the nervous system of in flies with decreased sedation in response to vapor from 50% ethanol. There was a significant overall effect of genotype on ST50s (one-way ANOVA, $p<0.0001$, n=8). ST50 values were not significantly different in w^{1118}, *v5597/+*, *elav-Gal4/+* or *elav-Gal4,v30034/+* flies (Bonferroni's, n.s.). *elav-Gal4/v5597* and *elav-Gal4,v30034/v5597* exhibited significantly higher ST50 values compared to relevant controls (*Bonferroni's, $p<0.05$ compared to *v5597/+* and *elav-Gal4/+*; **Bonferroni's, $p<0.05$ compared to *v5597/+* and *elav-Gal4,v30034*). *elav-Gal4/v5597* and *elav-Gal4,v30034/v5597* were not statistically distinguishable (Bonferroni's, n.s.). B. Knockdown of *mini-w* in the nervous system in flies with increased sensitivity to sedation from vapor from 50% ethanol. Overall, genotype had a significant effect on ST50s (one-way ANOVA, $p<0.0001$, n=8). ST50 values were indistinguishable in w^{1118}, *elav-Gal4/+* and

elav-Gal4,v30034/+, whereas the *ph- v50024/+* control was significantly different from w^{1118} (#Bonferroni's, $p<0.05$). *elav-Gal4;v50024* and *elav-Gal4/v30034;v50024* were not different from each other, but they were significantly different from their relevant controls (*Bonferroni's, $p<0.05$ compared to *elav-Gal4/+* and *v50024/+*; **Bonferroni's, $p<0.05$ compared to *elav-Gal4/+* and *elav-Gal4,v30034*).

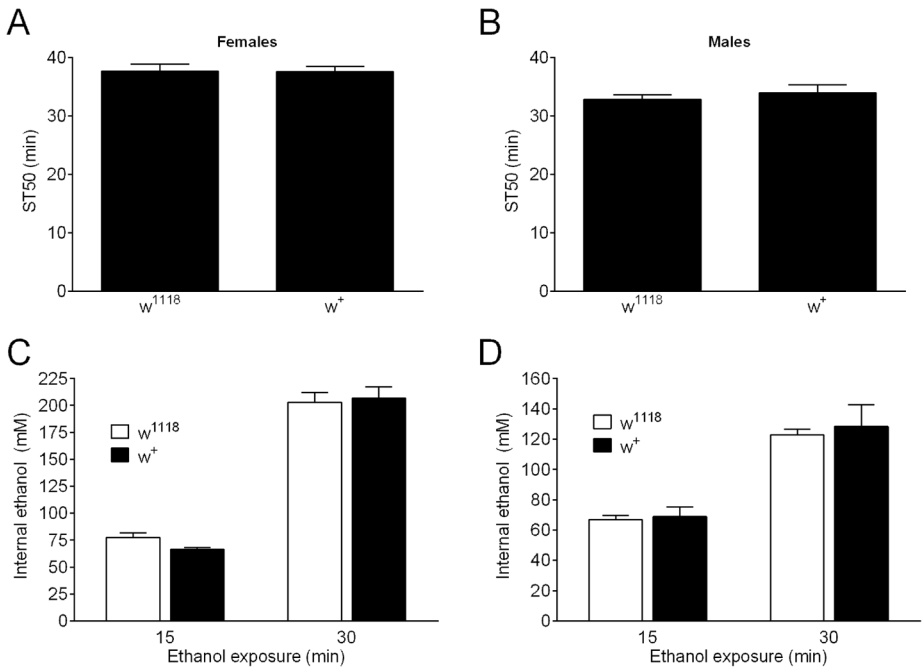

Figure 6. Ethanol sedation sensitivity and internal ethanol concentrations in *w* null and *w* wild-type flies

ST50 values in response to vapor from 50% ethanol were indistinguishable in *w* null (w^{1118}) and *w* wild-type (w^{+}) females (panel A, unpaired t-test, n.s., n=6 for w^{1118}, n=21 for w^{+}) or males (panel B, (unpaired t-test, n.s., n=10 per genotype). C and D. Internal ethanol concentrations in response to vapor from 50% ethanol were not distinguishable in w^{1118} and w^{+} females (C) and males (D), but were affected by duration of ethanol exposure (individual two-way ANOVAs; effect of *w* genotype, n.s.; effect of ethanol exposure time, $p<0.0001$; n=5 per genotype, sex and exposure time).

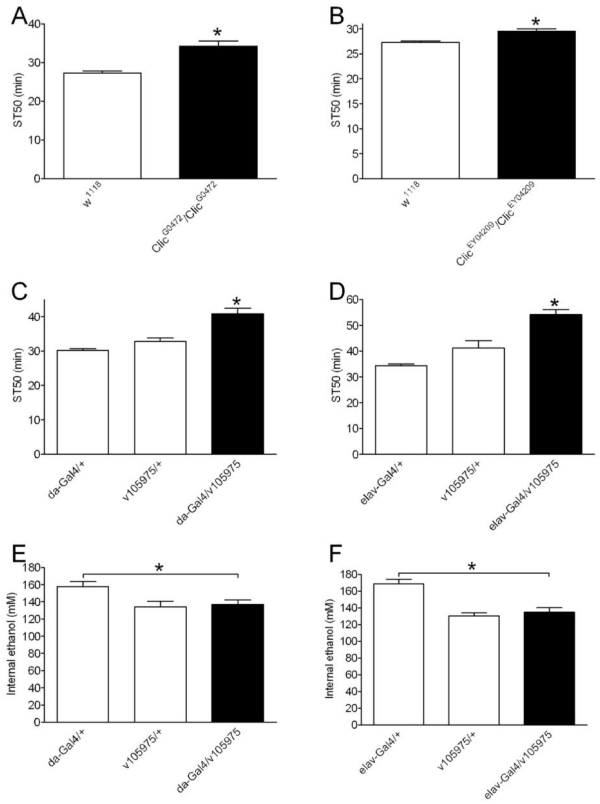

Figure 7. Mutations in and RNAi-mediated knockdown of *Clic* reduce ethanol sensitivity in ethanol sedation assays

ST50s were greater in homozygous $Clic^{G0472}$ (A) and $Clic^{EY04209}$ (B) transposon mutants (closed bars) than in w^{1118} controls (open bars) (*individual t tests, p≤0.027, n=10 per genotype) in ethanol sedation assays with vapor from 50% ethanol. Control and *Clic* mutant flies were reared at 20°C to circumvent homozygous lethality of the *Clic* alleles at 25°C. Ubiquitous (via *da-Gal4*, panel C, filled bar) or nervous system (via *elav-Gal4*, panel D, filled bar) expression of RNAi targeting *Clic* (*v105975*) lowered ethanol sensitivity compared to *Gal4/+* and *v105975/+* controls (open bars) (individual one-way ANOVAs, p<0.0001; *Bonferroni, p<0.05 compared to controls; n=8–10 per group). Internal ethanol concentrations were not consistently different in ubiquitous (E) and nervous system (F) *Clic* knockdown flies compared to Gal4 and v105975 controls (individual one-way ANOVAs; panel E, p=0.0288; panel F, p=0.0003; n=5; *Bonferroni's multiple comparisons test, p<0.05 compared to Gal4 controls). Controls are (A) w^{1118} in a Canton-S background, (B) *2202U*, (C) *WTB*, and (D) the progeny from *NPFR1-Gal4* or *NPFR1-RNAi* crossed to our standard w^{1118} strain.