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Genetic Analysis of Ethanol Sensitivity and Tolerance in Drosophila

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GENETIC ANALYSIS OF ETHANOL SENSITIVITY AND TOLERANCE IN
DROSOPHILA

A Thesis Submitted in Partial Fulfillment of the Requirements for
the Degree of Master of Science
at Virginia Commonwealth University.

by
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B.S., James Madison University, 2009

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July 2013
Abstract

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By Robin F. Chan, B.S.

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The genetic pathways influencing alcohol abuse and dependence are poorly characterized. Many critical discoveries about the interactions between ethanol-related behaviors and genetics have been made in the fruit fly *Drosophila melanogaster*. Coupling the statistical power of model organism studies to human association studies bolsters the analytical efficacy of these genomic approaches. A variety of behavioral assays are available for assessing behavioral responses to ethanol in *Drosophila*. However, we find our previously described eRING assay is influenced by the commonly used transgenic marker *mini-white*. We developed a Simple Sedation Assay (SSA) that is insensitive to the effects of *white* and *mini-white*. In SSAs, expression of endogenous wild-type *white* was not necessary for normal responses to ethanol. Neither expression nor RNAi-mediated knockdown of the transgenic *mini-white* influenced the effects of ethanol in flies. Critically, *mini-white* expression did not affect the phenotypes of flies with known alterations in ethanol sensitivity. Also, loss of function mutations in *Clic* show decreased sensitivity to ethanol in both eRING assays (as previously reported) and SSAs. Therefore, we explored the role of the known *Clic* interactors, TGF-β and ryanodine receptors. These studies were inconclusive but do not exclude the need for future work. Finally, using bioinformatic tools we constructed a mutli-species network of genes predicted to interact with *Clic*. Our RNAi screen against the *Clic* network serves as an important proof-of-concept and holds great potential for uncovering important therapeutic targets for alcohol use disorders.
Clarification of Contributions

Throughout the course of my graduate studies and the constructing of this thesis, I have benefited greatly from the assistance of many people. The volume of work presented here could not have been so comprehensive without significant contributions from the individuals acknowledged below:

Chapter II

The eRING data for the TGF-βR and Akap200 mutants was generated by Kristyn Sennett and Scarlett Coffman, respectively; eRING testing of Gal4 lines was performed in collaboration with Lara Lewellyn (Figure 3). Mike Grotewiel and Jacquie DeLoyht assisted in the preparation of flies for the experiment presented in Figure 4b. Characterization of the Simple Sedation Assay (Figures 6-8) was performed wholly by Lara Lewellyn and Matt Hewitt. Re-evaluation of the TGF-βR, Akap200, and Gal4 strains in SSAs (Figure 11) was performed in cooperation with Lara Lewellyn, Matt Hewitt, and Mike Grotewiel. Finally, backcrossing of the wild-type allele of white into our w^{1118} genetic background was performed by Lara Lewellyn.

Chapter III

Construction of the former Clic multi-species gene interaction network was conducted in conjunction with Mike Grotewiel, with assistance with Michael Miles. Finally, fly preparation and behavioral genetic screening of the initial Clic network was performed in cooperation with Lara Lewellyn and Matt Hewitt.
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I. Introduction

A. Alcohol Use Disorders

Alcohol use disorders (AUDs) have few cultural or geographic boundaries and are causal factors in a large number of diseases [1]. Within the general population, the lifetime risk for an individual to experience an episode of alcohol dependence is over 12% [2]. Interestingly, as much as 50% of the liability for developing an AUD is heritable [3-6] indicating a large genetic influence on alcoholism [7]. Although many studies have suggested the involvement of a large number of genes in alcoholism [8], few individual genes have been unambiguously associated with alcohol abuse. As in other areas of biomedical research, the identification and testing of candidate genes for AUDs is most readily accomplished in animal models of disease.

B. Assessing Behavioral Responses to Ethanol in Drosophila

With a scientific legacy spanning more than a century, the common fruit fly *Drosophila melanogaster* remains one the most highly utilized model organisms. The attractiveness of the fly model stems from their ease of handling, relatively low cost, rapid generation time, and moderately complex organ systems. The extensive and well refined catalog of genetic tools available for use in *Drosophila* also greatly expedites investigations in the fly model. As numerous human and fly genes display conserved biological functions [9], the extensive characterization of the genetics and biochemical pathways of *Drosophila* positions it as an ideal model for investigating human AUDs. Notably, an estimated 75 percent of human protein-coding genes associated with disease have an identifiable orthologs in the fly genome [10].
Critically, flies exhibit behavioral responses to ethanol that mirror those observed in mammals [11, 12]. Low doses of ethanol induce locomotor stimulation which transitions into full sedation as the dosage of ethanol increases. Tolerance to these sedative effects of ethanol also develops in flies after repeated exposure [12]. With the advent of transposon-mediated mutagenesis and transgenesis [13] it became possible to use the fly to rapidly screen for genes that influence behavioral responses to ethanol [14-16]. Thus, various assays have been developed over the years to gauge the major behavioral changes that manifest in flies following exposure to ethanol. In general, the most commonly used assays for acute sensitivity to ethanol measure alterations in posture or locomotor activity.

Loss of postural control is a hallmark of the sedation resulting from a high dose of ethanol. The oldest and most complex device for measuring loss of postural control is the inebriometer. Constructed of repeating mesh baffles, contained inside a large vertical glass column, use of the inebriometer is in effect column chromatography for intoxicated flies [17]. Approximately 100 flies are placed in the upper chamber of the column as ethanol vapor flows from the top to the bottom. As flies begin to sedate and lose postural control, they tumble down the length of the column and finally elute from the bottom. The average time required for flies to elute from the inebriometer, the mean elution time, is a function of their sensitivity to ethanol.

Other assays favor measuring changes in postural control by direct visual observation. Loss of postural control in the presence of ethanol can be gauged by scoring for flies that have lost their ability to return to an upright position following physical agitation (loss of righting reflex) [18-22].

Assessing the locomotion of flies following treatment with ethanol has been most accurately accomplished by using a computerized video tracking system [23]. Distinct modes of locomotor activity are observed in these experiments during the course of
ethanol exposure. Initially, an olfactory response induces locomotor activation following the introduction of ethanol vapor [23, 24]. Following this transient startle, flies become hyperactive then akinetic and sedated, as their internal ethanol concentrations rise. Locomotor activity can also be evaluated by recording changes in the innate negative geotaxis reflex of flies. Reductions in the ability of flies to climb following agitation are seen in the presence of ethanol and are the basis for the eRING assay [25].

The development of tolerance can be observed as a reduction in the effects of ethanol in these behavioral assays during a subsequent re-exposure. The two forms of functional tolerance most readily observed in flies are rapid and chronic tolerance [11, 12, 26]. Rapid tolerance peaks a few hours after the initial exposure and represents pharmacodynamics changes in the nervous system [12, 26]. Chronic treatment with ethanol results in long-term reductions in ethanol sensitivity that requires protein synthesis, and is likely due to ethanol-induced changes in gene regulation [26].

C. The Genetics of Ethanol Sensitivity and Tolerance in Drosophila

Genetic screens in *Drosophila* have identified a multitude of genes important in modulating behavioral responses to ethanol. Strikingly, studies in *Drosophila* have highlighted many distinct yet overlapping classes of genes as modulators of ethanol-related behaviors (ERBs). The gene families with the greatest evidence of influencing responses to ethanol are those involved in neurotransmission, cAMP signaling, growth factor signaling, and cell adhesion (Table 1). Importantly, many orthologs of these *Drosophila* genes have also been shown to have effects on ethanol responses in other species.

In mammals, ethanol has many direct pharmacological targets including the γ-aminobutyric acid (GABA), glycine, and *N*-methyl-]*D*-aspartate (NDMA) receptors [27].
Unsurprisingly, genes involved in dopaminergic and GABAergic signaling have been shown to influence ERBs in flies [28-30]. However, studies in *Drosophila* also frequently identify novel or previously uncharacterized genes that modulate ethanol sensitivity and tolerance.

Synaptic strength is often associated with the density and morphology of dendritic spines, the major sites of excitatory post-synaptic input. The growth and organization of these dynamic structures depends greatly on the layout and stability of their actin cytoskeleton. The Rho family of small GTPases are major regulators of the actin cytoskeleton, and are inhibited by GTPase-activating proteins (GAPs). Mutant alleles of the gene for one such protein, *RhoGAP18B*, result in increased ethanol resistance [18] and decreased voluntary consumption of ethanol [11] in flies. Additionally, the small GTPases Arfaptin, Arf6, and Rac1 interact with one another, and in cooperation with RhoGAP18B modulate the behavioral effects of ethanol [31]. Mice lacking *Eps8*, another modulator of actin remodeling, are more resistant to the sedative effects of ethanol [32]. Interestingly, increased ion current through NMDA receptors was observed in *Eps8* knockout mice [32] highlighting how changes in non-receptor protein activity can indirectly affect ethanol’s effects on wild-type ion channels.

The development of tolerance to ethanol in mammals is typically associated with the induction of stress responses and requires intact noradrenergic neurons [33]. While insects lack noradrenaline, octopamine fulfills an analogous neuromodulatory role in invertebrates [34]. Therefore, Scholz *et al* (2000) reasoned octopamine may influence ethanol-related behaviors in flies. Indeed, *Drosophila* tyramine β-hydroxylase mutants, which lack the ability to synthesize octopamine, display impaired rapid ethanol tolerance [12]. Additionally, induction of cellular stress responses via heat-shock can induce tolerance to ethanol in naïve flies [35, 36].
Overall, there is considerable overlap of the biochemical pathways implicated in ERBs of flies and rodents. Additionally, a number of genes found to affect ERBs in Drosophila are also significantly associated with alcohol abuse and dependence in humans (Table 1). Collectively, these studies establish the utility of the fly model for investigating genes that have conserved effects on ethanol-related behaviors, including genes implicated in human AUDs.

<table>
<thead>
<tr>
<th>Function</th>
<th>Fly Gene</th>
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<td>ARL6IP5</td>
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</table>

Genes presented are those that are strongly implicated in behavioral studies in Drosophila and in at least one human genome-wide association study.

D. Genomic & Cross-Species Investigations

Perhaps the most exciting frontier in AUD research is the emergence of whole genome and cross-species approaches that greatly facilitate candidate gene
investigation and discovery. The development of next-generation sequencing and microarray technologies has made it possible to rapidly screen for polymorphisms and changes in gene expression associated with alcohol abuse. These tools have allowed for identification and testing of many novel genes with roles in ERBs in multiple model organisms. Most importantly, studies utilizing cross-species collaborations extend the experimental flexibility and statistical power of model organism studies into human association studies. The analytical power of this approach is illustrated by studies on the Clic4/Clic genes that influence ethanol sensitivity in flies, worms and mice [64].
II. The Influence of *white* and *mini-white* on Behavioral Responses to Ethanol

A. Introduction

A common feature of most genetic studies in *Drosophila*, including those focused on ethanol-related behaviors 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 [65, 66]. White protein localizes to the endosomes of pigment cells where it cooperates with Brown and Scarlet to mediate the intracellular transport of guanine and tryptophan metabolites [67, 68]. Thus, expression of *white* in wild-type flies results in the development of red colored eyes. In contrast, null mutations of *white* lead to a complete loss of eye pigmentation that appears white in color.

The *mini-w* mini-gene cassette within many currently used *Drosophila* transformation vectors originates from the *pW6* [69] and *pCaSpeR* [70] P-element transformation vectors. The *mini-w* cassette from *pCaSpeR* (*w*+*mC*) consists of ~250 bp of upstream and ~600 bp of downstream regulatory *white* sequence, with most of the 5’ large first intron removed. In the *pW6* vector, the minimal *white* promoter is replaced with the *Hsp70* minimal promoter (*w*+*mw.hs*) (Figure 1).
Transformation of white null mutants with these vectors, or their many derivatives, rescues eye pigmentation as a result of production of White protein. The convenient nature of the \(w\) eye color phenotype has made mini-\(w\) a routinely used marker for transgenesis in Drosophila. Currently, there are 38,828 fly lines catalogued on FlyBase that carry a mini-\(w\) construct [71]. Although other markers for transgenesis are used in Drosophila (e.g. yellow and GFP [15]), the mini-\(w\) marker is the most commonly encountered in genetic studies in flies.

The \(w\) gene product is highly conserved among many insects and is structurally related to the ABCG1 in humans that is associated with multiple mental health disorders [72, 73]. White is important for the function of organ systems outside the eye in Drosophila, as it is abundant in the prepupal fat body (functional analog of the liver) and the adult malpighian tubules (excretory organ) [74]. Furthermore, several recent studies have shown that endogenous \(w\) or mini-\(w\) influences multiple non-visual neural

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**Figure 1:** Schematic of white and mini-white genes. The protein coding sequence of wild-type endogenous white (orange boxes) and transgenic mini-white constructs (pink and red boxes) is comprised of six exons (numbered boxes). The large first intron is truncated in mini-white constructs. In hs-mini-white \((w^+_{mW:hs})\) the ~250bp minimal white promoter found in mini-white \((w^+_{mC})\) is replaced by the hsp70 minimal promoter (blue square).
processes. Heads of \( w \) null mutants have altered levels and localization of the biogenic amines dopamine, serotonin, and histamine [75] and flies deficient in \( w \) display abnormal responses to volatile anesthetic gases [76]. Endogenous \( w \) transcripts are also found in fly heads from which pigment cells have been genetically ablated [76], also supporting a role for \( white \) that is independent of vision. Additionally, \( mini-w \) over-expression induces male-male courtship behavior in flies [68, 77, 78] and expression of \( white \) or \( mini-w \) is necessary for ethanol-induced male-male courtship behavior [79]. Taken together, it is clear that expression of \( white \) and/or \( mini-w \) can have significant effects on the neurochemistry and behavior of \( Drosophila \).

Given the use of the fly as a model for ethanol-related behaviors and the widespread use of \( mini-w \) as a transgenic marker in flies, we explored the contribution of \( mini-w \) and endogenous \( w \) expression to ethanol sedation sensitivity and rapid tolerance. We found that ethanol sensitivity measured in our previously described eRING assay [25] correlated with \( mini-w \) expression from several independent transposon insertions, making this behavioral assay difficult to use for genetic analyses of ethanol sensitivity. To circumvent the experimental confounds with \( mini-w \) in eRING assays, we developed a simple sedation assay (SSA) based in part on previous reports from other laboratories [20, 22, 42]. We found that sensitivity to ethanol sedation in SSAs was time- and dose-dependent as expected and that rapid tolerance to ethanol was readily observed in SSAs. We also found that neither \( mini-w \) nor endogenous \( white \) significantly affected ethanol sensitivity, rapid ethanol tolerance, internal ethanol concentrations or locomotor behavior related to SSAs. Importantly, ethanol sensitivity measured in SSAs is sensitive to mutation of \( Clie \) and expression of RNAi against two other genes (\( cnx14D \) and \( ph-p \)) identified in reverse genetic screens. Considering these findings and the ability to multiplex testing of several genotypes in parallel in SSAs, the assay is well-suited for
genetic studies on the behavioral responses to acute ethanol exposure in *Drosophila*, including studies using transgenic strains marked with mini-w.

**B. Results and Discussion**

*eRING Results are Influenced by mini-w and white*

Our laboratory previously described eRING (ethanol rapid iterative negative geotaxis) as an assay for measuring ethanol sensitivity in *Drosophila* [25]. 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 marker contained 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. Expression of mini-w, which varies greatly in different transposon strains due to well-documented position effects [80, 81], was ranked in our studies by eye color (w+-rank) by a single experimenter blind to genotype (Figure 2).

The series of flies assessed in eRING assays harbored (i) transposon insertions in the Akap200 locus, (ii) transposon insertions in three TGF-β receptor genes (*thickveins* (*tkv*), *wishful thinking* (*wit*), baboon (*babo*)), and (iii) several different Gal4 transgenes, all marked with mini-w. Expression of mini-w from Akap200 and TGF-β receptor transposon insertions correlated with ethanol sensitivity in eRING assays (Figure 3), but not with internal ethanol concentration, expression of Akap200 or TGF-β receptor genes, or locomotor behavior in the absence of ethanol (not shown). We also found a similar trend, albeit non-significant, between mini-w expression from Gal4 transgenes and ethanol sensitivity in eRING assays (Figure 3).
Figure 2: Eye color phenotypes of wild-type, w null, mini-w transformed, and w knockdown strains. All images are of female flies. w null (w¹¹¹⁸) mutants (A) show an absence of eye pigmentation in contrast to wild-type (w*)(B). Heterozygous Gal4 lines containing a single copy of mini-w display a range of eye colors due to varied levels of marker expression (C-I). Uninduced w³⁰⁰³³/+ and w³⁰⁰³⁴/+ adults show mild mini-w expression (J & K). The eye color in flies with w-RNAi driven by elav-Gal4 (elav-Gal4,w³⁰⁰³³, elav-Gal4/w³⁰⁰³⁴, and the recombinant chromosome elav-Gal4,w³⁰⁰³⁴) (L & M) are indistinguishable from w¹¹¹⁸ mutants.

Figure 3: Ethanol sensitivity correlates with eye color in eRING assays. A. Flies harboring Gal4 drivers or transposon insertions in Akap200 or the TGFβR genes tkv, wit and babo were ranked by eye color (w+ rank, X axis) and tested in eRING assays for sedation to ethanol vapor. T50 values from eRING assays using 50% ethanol (Akap200) or 20% ethanol (TGFβR) correlated with w+ rank (Akap200, Pearson r=0.9369, p=0.0006; TGFβR, Pearson r=0.8056, p<0.0001). There was a trend toward correlation between T50 values from eRING assays and w+ rank in Gal4 lines (Pearson r=0.6104, p=0.108). Akap200 and TGFβ lines tested were: Akap200 (EP2254, c01373, d01782, d03938, d07255, EY04645 and EY12242), tkv (7, 8, d07811, f02766, f03305, c06013 and KG05071), wit (d02492, e00566 and e01243) and babo (c04263, c05710, k16912). Gal4 lines tested were da-Gal4/+, mef2-Gal4/+, Appl-Gal4/+, Actin5cGal4/+, GMR-Gal4/+, 24B-Gal4/+, and elav-Gal4/+. See Figure 2.
These trends and correlations strongly suggest that mini-w expression reduces initial sensitivity to ethanol as determined by eRING. Therefore, we predicted that knockdown of mini-w would in turn increase the sensitivity of flies to ethanol. To test this hypothesis, we used nervous system Gal4 (elav\textsuperscript{C155})\textsuperscript{[82]} to drive expression of a UAS-RNAi transgene (\textit{w\textsuperscript{30034}}) that targets mini-w. Nervous system expression of \textit{w\textsuperscript{30034}} led to an eye color phenotype indistinguishable from \textit{w\textsuperscript{1118}} null flies (Figure 2), confirming that expression of \textit{w\textsuperscript{30034}} strongly knocked-down mini-w derived from the Gal4 and UAS transgenes themselves. When tested in eRING, the sensitivity of these mini-w knockdown flies was virtually identical to \textit{w} null mutants, and significantly more sensitive to ethanol than the mini-w expressing \textit{elav-Gal4/+} and \textit{w\textsuperscript{30034}/+} controls (Figure 4a). Thus, \textit{mini-white} expression greatly influences initial sensitivity to ethanol in eRING assays.

Finally, as both mini-w and endogenous white share identical coding sequences, we expected that wild-type endogenous white expression would similarly affect ethanol sensitivity. The wild-type allele of \textit{w} (\textit{w+}) from Canton-S was backcrossed into our \textit{w} null (\textit{w\textsuperscript{1118}}) control strain for eight generations. Assessed via eRING, ethanol sensitivity was reduced nearly two-fold in flies homozygous for wild-type white (\textit{w+}) compared to \textit{white} null mutants (Figure 4b).

These results demonstrate that expression of both mini-w markers and endogenous white influence sensitivity to ethanol in this behavioral paradigm. This apparent confound represents a significant limitation to the utility of the eRING assay given the widespread use of mini-w as a genetic marker in \textit{Drosophila}. 
We pursued an alternative behavioral paradigm to circumvent the apparent white and mini-w confounds in eRING assays (Figure 3 & 4). We reasoned that an ideal behavioral assay would be easy to perform, reproducible, relatively inexpensive and sensitive to the dose of ethanol, but would not sensitive to expression of mini-w.

**Development of the SSA**

![Simple Sedation Assay testing vial arrangement.](image)

Flies are placed into food vials, ethanol is added to the cellulose acetate Flug, and the vials are sealed with a rubber stopper. B. Vials are tested in ascending numerical order in groups of 4. Agitation and scoring for sedation of each vial within a group of 4 is offset by 5 seconds. Each row (group of four) is offset from the next row by 1 minute. Additional details are provided in Materials and Methods for full description.
We developed a Simple Sedation Assay (SSA) with these considerations and the work of others in mind [20, 22, 42]. In SSAs, 10 flies are placed in a 9.5 cm tall plastic food vial and trapped in the vial with a cellulose acetate Flug (Figure 5A). Ethanol solution is the added to top (exposed side) of the Flug and the vial is immediately sealed with a rubber stopper (Figure 5A). Thereafter, at 6-minute intervals flies are gently tapped to the bottom of the vial and then visually scored for their ability to maintain postural control and/or coordinated locomotion in the continuous presence of ethanol vapor. SSAs can be used to test multiple replicates of several genotypes in parallel (Figure 5B). Each vial of flies corresponds to n=1 and we have found that an individual experimenter can readily test 24 vials simultaneously in a single experiment.

The primary data from SSAs are the percentages of non-sedated flies as a function of ethanol exposure time (Figure 6A and 6B). 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 [20]. In an effort to expedite data analysis, we explored interpolating ST50 values from curve fits of our SSA time-course data. We found that third-order polynomials fit SSA time-course data closely (R^2 = 0.96±0.001, n=1221) and better than first-, second- or fourth-order curves. We therefore use ST50 interpolated from third-order polynomial curves as a measure of ethanol sensitivity in SSAs. Note that lower and higher ST50s indicate increased and blunted ethanol sensitivity, respectively.

To determine if flies are sensitive to ethanol dose in SSAs, 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 6A, 6B, 7A, 8A, and 8B). In contrast, exposure to vapor from increasing concentrations of ethanol progressively hastened time-dependent sedation (females, Figure 6A; males, 6B) and therefore also decreased ST50s (females, Figure 6C; males, 6D). Exposure to vapor
Figure 6: Exposure to ethanol vapor 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 a significant effect 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<0.0002, 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).
from increasing concentrations of ethanol also increased the internal ethanol content of flies (females, Figure 6E; males, 6F), demonstrating that sedation in the SSA is dose-dependent.

Although in some of our initial studies we noticed that ST50s in w1118 females and males appeared to be different when tested in separate experiments (Figure 6C and 6D), we found that males and females performed indistinguishably when tested side-by-side (Figure 7A and 7B). Thus, all flies compared in behavioral and molecular experiments are grown in parallel and tested on the same day. Flies in SSAs loose a comparable amount of body mass when exposed to vapor from water or ethanol (Figure 7C), indicating that sedation in the presence of ethanol vapor is not due to dehydration.

Rapid tolerance is defined as blunted ethanol sensitivity to a second exposure to the drug following recovery from an earlier initial exposure [11, 12]. Control w1118 flies became sedated during both a first (E) and second (EE) ethanol exposure separated by four hours of recovery in SSAs, but were significantly less sensitive during the second ethanol challenge (females, Figure 8A and C; males, Figure 8B 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 8A-D), indicating that blunted ethanol sensitivity in EE flies requires multiple exposures to the drug. Internal ethanol concentrations were indistinguishable during a first and second ethanol exposure (females, Figure 8E; males, Figure 8F). We conclude that the blunted ethanol sensitivity during the second ethanol exposure in SSAs is due to altered pharmacodynamic properties of the drug and therefore that this change in behavior represents rapid tolerance.
Figure 7: Ethanol sedation is not associated with ethanol-induced dehydration. A. Time-dependent ethanol sedation in female (circles) and male (squares) w^{1118} control flies. Time and ethanol had significant effects on the percent active flies and there was an interaction between the two factors as expected (two-way ANOVA; effect of time, p<0.0001; effect of ethanol treatment, p<0.0001; time x ethanol interaction, p<0.0001; n=10 per sex per ethanol treatment). B. ST50 values derived from the data in panel A were not distinguishable in female and male flies exposed to ethanol (unpaired t test, n.s., n=10 per sex). C. Percent loss in total wet weight was greater in females than males, but was not different in flies exposed to water versus ethanol vapor in SSAs in either sex (two-way ANOVA; effect of sex, p<0.0001; effect of ethanol versus water, n.s.; sex x ethanol interaction, p=0.0144; n=10 per sex and treatment).
**Figure 8: Rapid tolerance in control flies.** Data are from \( w^{118} \) control female (A, C and E) and male (B, D and F) flies exposed to vapor from 50% ethanol (E) or water (W) as indicated. A and B. Sedation time-courses from flies exposed once to vapor from water (W), exposed once to vapor from ethanol (E), exposed to water vapor, allowed to recover for 4 hours, then exposed to ethanol vapor (WE), and exposed to ethanol vapor, 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.).
**Endogenous white and SSAs**

We first examined the effects of endogenous w expression on sensitivity and rapid tolerance to ethanol in SSAs. We found no differences in initial ethanol sensitivity in w null and w wild-type females (Figure 9A) or males (Figure 9B). The development of rapid tolerance to ethanol was similarly unaffected by w genotype in either sex (Figure 10A and 10B). Additionally, there were no significant differences in internal ethanol concentrations (Figure 9C and 9D) or negative geotaxis before or after a mock SSA (Figure 10C and 10D) in w<sup>1118</sup> and w+ flies. In contrast to eRING, these results indicate that endogenous white has no discernible effect on ethanol sensitivity, rapid ethanol tolerance, ethanol kinetics, or negative geotaxis related to SSAs.

**Figure 9: Ethanol sedation sensitivity and internal ethanol concentrations in w null and w wild-type flies.** ST50 values were indistinguishable in w null (w<sup>1118</sup>) and w wild-type (w+) females (panel A, unpaired t-test, n.s., n=6 for w<sup>1118</sup>, n=21 for w+) or males (panel B, unpaired t-test, n.s., n=10 per genotype). C and D. Internal ethanol concentration were not distinguishable in w<sup>1118</sup> and w+ females (C) and males (D), but were affected by time of exposure to 50% ethanol (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).
Manipulation of mini-w in SSAs

We next used the SSA to address the influence of mini-w expression on ethanol sensitivity and rapid tolerance. We first repeated assessment of initial sensitivity in Akap200 and TGF-β receptor transposon insertion genotypes, and several different Gal4 transgenic lines. We found no correlation between ST50s from SSAs and expression of mini-w in any of the three groups of transgenic animals (Figure 11). In contrast to the data from eRING assays (Figures 3 & 4b), these results suggest that mini-w does not influence ethanol sensitivity measured in SSAs. To validate the ability of the SSA to detect differences in ethanol sensitivity, we also tested ClicG0472/G0472 mutants previously

Figure 10: Rapid ethanol tolerance and negative geotaxis in w null and wild-type flies. A and B. The development of rapid tolerance to ethanol sedation was not significantly different between w^{1118} and w^{*} female (A) or male (B) flies (individual unpaired t-tests, n.s., n=8-10 per genotype and sex). C and D. Negative geotaxis was not affected by w genotype or by exposure to mock SSAs with water (individual two-way ANOVA; effect of genotype, n.s.; effect of mock SSA, n.s., n=6 per genotype and treatment group), although there was an interaction between genotype and mock SSA in females (Panel C, p=0.0065).
found to have decreased sensitivity to ethanol in eRING studies [83]. In the SSA, deficiency in Clic resulted in a significantly increased ST50 (Figure 12) as predicted.

**Figure 11: Ethanol sensitivity in SSAs does not correlate with eye color.** Flies harboring Gal4 drivers or transposon insertions in Akap200 or the TGFβR genes tkv, wit and babo were ranked by eye color (w+ rank, X axis) and tested in SSAs for sedation to ethanol vapor. ST50 values from SSAs using 50% ethanol did not correlate with w+ rank in Gal4, Akap200 or TGFβR lines (Pearson r=-0.0300 to -0.3307, p=0.5868 to 0.9302). Akap200 and TGFβ lines tested were: Akap200 (EP2254, c01373, d01782, d03938, d07255, EY04645 and EY12242), tkv (7, 8, d07811, f02766, f03305, c06013 and KG05071), wit (d02492, e00566 and e01243) and babo (c04263, c05710, k16912). Gal4 lines tested were da-Gal4/+, mef2-Gal4/+, Appl-Gal4/+, Actin5cGal4/+, GMR-Gal4/+, 24B-Gal4/+ and elav-Gal4/+. See Figure 2.

**Figure 12: Acute ethanol sensitivity in SSAs of flies deficient in Clic.** Flies homozygous for the loss of function Clic allele G0472 had significantly higher ST50 values as compared to w1118 controls (two-tailed t-test, p<0.0001, n=8). Both Clic mutants and w1118 controls were grown at 20°C.
To further test the role of *mini-w* in SSAs, we again used nervous system Gal4 to drive expression of two UAS-RNAi transgenes (*w* \(^{30033}\) and *w* \(^{30034}\)) to knockdown *mini-w*. Nervous system expression of the additional *w* \(^{30033}\) RNAi transgene resulted in knockdown of *mini-w* similar to that observed following *w* \(^{30034}\) expression (Figure 2 L&M).

ST50s from SSAs was not significantly different in *w* \(^{1118}\), *elav-Gal4/+*, *w* \(^{30033}+/+\) and *w* \(^{30034}+/+\) controls (Figure 13A), consistent with our other experiments with *mini-w* in SSAs (Figure 11). Ethanol sensitivity in flies expressing *w* RNAi in the nervous system (*elav-Gal4;w* \(^{30033}\) and *elav-Gal4/w* \(^{30034}\)) was significantly increased compared to the *elav-Gal4/+* control, but not compared to the *w* \(^{30033}+/+\) or *w* \(^{30034}+/+\) controls (Figure 13A). Internal ethanol concentrations were comparable in all control and *w* knockdown strains tested (Figure 13B). Additionally, rapid tolerance developed in all control and *w* knockdown groups (Figure 14A). Although rapid tolerance was slightly blunted in *elav-Gal4/w* \(^{30034}\) knockdown flies compared to *elav-Gal4/+* and *w* \(^{30034}\) controls, this decrease in rapid tolerance was not found in *elav-Gal4;w* \(^{30033}\) knockdown flies (Figure 14A).

Finally, we observed no consistent effect of *mini-w* or knockdown of *mini-w* on negative geotaxis in naïve flies or in flies exposed to mock SSAs (Figure 14B and 14C). 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 alter ethanol sedation sensitivity, rapid tolerance to ethanol, ethanol uptake/metabolism or locomotor ability in flies as measured in or related to SSAs.
Figure 13: Ethanol sedation sensitivity, internal ethanol concentrations and RNAi-mediated knockdown of mini-w in females. Expression of w RNAi transgenes (w<sup>30033</sup> and w<sup>30034</sup>) were driven in the nervous system by elav-Gal4. A. Knockdown of mini-w in the nervous system and initial sensitivity to ethanol. Genotype had a significant overall effect on ST50 values (one-way ANOVA, p=0.0008, n=8). ST50 values in the w<sup>1118</sup>, elav-Gal4/+, w<sup>30033</sup>/+ and w<sup>30034</sup>/+ genotypes were not statistically different (Bonferroni’s, n.s.). ST50 values in elav-Gal4/w<sup>30033</sup> and elav-Gal4/w<sup>30034</sup> knockdown animals were greater than in elav-Gal4/+ (Bonferroni’s, *p<0.05, **p<0.01) but were not significantly different from w<sup>30033</sup>/+ or w<sup>30034</sup>/+ controls (Bonferroni’s, n.s.). B. Internal ethanol concentration of nervous system mini-w knockdown flies after 30 minutes of exposure to ethanol vapor. 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 14: Nervous system w RNAi, rapid ethanol tolerance and negative geotaxis. A. Knockdown of mini-w in the nervous system and rapid tolerance to ethanol. Overall, genotype had a significant effect on rapid tolerance (Kruskal-Wallis ANOVA, p<0.0001, n=18 per genotype). Rapid tolerance in elav-Gal4/w<sup>30034</sup> flies was significantly decreased relative to elav-Gal4/+ and w<sup>30034</sup>/+ controls (*Dunn’s multiple comparison test, p<0.05). B and C. Negative geotaxis was significantly affected by genotype (individual two-WAY ANOVAs; panel B, p<0.0001; panel C, p=0.0026; n=6 per genotype and treatment). Exposure to mock SSAs affected negative geotaxis in panel B (p=0.0003), but not in panel C (n.s.). There were no interactions between genotype and mock SSA exposure (n.s.). B. Naïve w<sup>1118</sup> was different from elav-Gal4/+ (*Bonferroni’s, p<0.05) and naïve and mock SSA-exposed elav-Gal4/w<sup>30033</sup> was different from elav-Gal4/+ and w<sup>30034</sup>/+ (**Bonferroni’s, p<0.05). C. Naïve elav-Gal4/w<sup>30034</sup> was different from elav-Gal4/+ and w<sup>30034</sup>/+ (*Bonferroni’s, p<0.05). All other pair-wise comparisons in panels B and C were not significant.
The data in the preceding experiments strongly indicate that mini-w does not impact behavioral performance in SSAs of flies with essentially normal ethanol sensitivity. However, we reasoned that mini-w might have subtle effects on SSAs performance that could be revealed in flies with altered baseline ethanol sensitivity. To test this hypothesis, we co-expressed w RNAi in conjunction with UAS-RNAi transgenes against cnx14D (cnx14D5597) or ph-p (ph-p50024). Expression of the cnx14D and ph-p UAS-RNAi transgenes (identified in a reverse genetic screen - see chapter IV) blunt and increase ethanol sensitivity, respectively (Figure 15A and 15B). To achieve coincident expression of RNAi against of w and either cnx14D or ph-p, we generated flies containing a recombinant X chromosome harboring an elav-Gal4 driver and the w30034 RNAi transgene in cis. Eye pigmentation in flies with this recombinant chromosome (elav-Gal4,w30034) is indistinguishable from w1118 null flies (Figure 2M), indicating strong knockdown of mini-w. Ethanol sensitivity in SSAs was statistically indistinguishable in w1118, elav-Gal4,w30034/+; elav-Gal4/+ and cnx14D5597/+ controls (Figure 15A). Expression of cnx14D5597 with elav-Gal4 led to the expected increase in ST50, but importantly this phenotype was not significantly affected by coincident knockdown of mini-w (Figure 15A). Similarly, in an independent set of experiments we found that ethanol sensitivity in w1118; elav-Gal4,w30034/+; and elav-Gal4/+ controls were comparable and that the increased sensitivity of flies expressing ph-p50024 RNAi was not affected by concurrent knockdown of mini-w (Figure 15B). Our interpretation of these data is that loss of mini-w expression has no significant effect on the phenotypes of flies with increased or decreased baseline sensitivities to ethanol.

Conclusions

We have developed the SSA as a behavioral paradigm for measuring ethanol sensitivity and rapid ethanol tolerance in Drosophila. The assay is sensitive to the dose
of ethanol used, a previously reported mutation in Clic, and expression of RNAi targeted against cnx14D and ph-p. Critically, in contrast to eRING assays, performance in SSAs is not significantly affected by mini-w or endogenous w expression. Our studies indicate that the SSA can be used as an experimental platform for probing the genetic basis for ethanol sensitivity and tolerance using many existing transgenic reagents for manipulating the fly genome, including those containing the widely used marker mini-w.
III: *Clic* Interactors as Possible Modulators of Ethanol Sensitivity and Tolerance

A. Introduction

The *chloride intracellular channel* (*Clic*) family of genes is highly conserved across animal species. The *Clic* family consists of six paralogs (*Clic1-6*) in mammals, two (*exc-1* and *exc-4*) in nematodes, and one locus in (*Clic*) in flies. Of these genes, the biochemistry of the product of mammalian *Clic4* is the best characterized. Like others in the family, CLIC4 is a putative intracellular anion channel [84] and is highly expressed in kidney, liver, brain. Interestingly, CLICs appear to exist in both a membrane bound and soluble conformation that is under control of redox conditions within the cell. Containing an N-terminal glutathione S-transferase domain and a nuclear localization sequence on the sixth α-helix, CLICs appear to have a diverse array of cellular functions [85]. CLICs are implicated in regulating multiple biochemical pathways including those of A-kinase anchoring proteins [86], cytoskeletal elements [87], ryanodine receptors [88, 89], and growth factor signaling [90].

Transcriptome analysis shows that ethanol induces expression of *Clic4* in the brains of mice [83, 91]. Importantly, partial loss of function of *Clic* in *Drosophila* leads to decreased sensitivity to ethanol. Additionally, loss of *exc-1* and *exc-4* in *C. elegans* and viral over-expression of *Clic4* in mouse brain also results in changes to ethanol responsive behaviors in these animals [83]. Considering the numerous biochemical pathways in which CLICs participate, genes encoding products known to interact with CLICs may also play roles in ethanol responses. Accordingly, we chose to investigate ryanodine receptors and the TGF-β pathway, both known to interact with *Clic*, as potential modulators of initial sensitivity and rapid tolerance to ethanol. Furthermore, we constructed a network of genes predicted to interact with *Clic*, and have demonstrated novel roles for many genes in ethanol related behaviors.
B. Ryanodine Receptors

Introduction

Intracellular Ca^{2+} is a key secondary messenger and is essential for the function of excitable cells. The endoplasmic reticulum (ER) serves as the major site of intracellular calcium storage in cells. Ca^{2+} stored in the lumen of the ER is released primarily through inositol 1,4,5-triphosphate receptors and the ryanodine receptors (RyRs). Depending on cell type, ryanodine receptors respond to action potentials through physical coupling to transmembrane L-type Ca^{2+} channels or via Ca^{2+} induced Ca^{2+} release (reviewed in [92]).

Three mammalian isoforms have been experimentally detected and vary in expression by tissue type. RYR1 and RYR2 are expressed most highly in skeletal muscle and cardiac muscle, respectively. In contrast, RYR3 is expressed primarily in the brain, and has been implicated in depression [93], memory [94], neurodegenerative disease [95], and has also been significantly associated in alcohol use disorders in humans [51]. Worms deficient in the ryanodine receptor ortholog unc-68 also demonstrate augmented behavioral responses to ethanol (J. Bettinger & A. Davies personal communication). In mammals, Ca^{2+} current through RyR2 in cardiac muscle is modulated by CLIC2. During oxidative conditions CLIC2 inhibits RyR2 current, whereas it increases RyR2 current in an environment of high reduction potential [88, 89].

Noting the evidence of ryanodine receptor involvement in responses to ethanol in worms and humans, and its relationship with CLICs, we hypothesized that Drosophila RyRs (encoded by Rya-44F) influences ethanol sensitivity and rapid tolerance. We therefore performed behavioral testing on flies with altered Rya-44F expression.
Results & Discussion

Initial sensitivity and rapid tolerance to ethanol were determined for flies carrying transposon insertions (d03686 and k04913) in the Rya-r44F locus using SSAs. Control w^{1118} flies did not differ from Rya-r44F^{d03686/+} or Rya-r44F^{d03686/k04913} mutants in ST50s, while Rya-r44F^{k04913/+} and Rya-r44F^{d03686/d03686} flies were slightly more sensitive to ethanol (Figure 16A). Rapid tolerance was not changed significantly in heterozygous Rya-r44F mutants, but was greatly blunted in double mutants (Figure 16B).

![Graph A and B](#)

**Figure 16: Initial sensitivity and rapid tolerance to ethanol in Rya-r44F mutant flies.** A. Genotype had a significant overall effect on ST50 (one-way ANOVA, p<0.0001, n=20). ST50 values were not different between w^{1118} controls, Rya-r44F^{d03686/+} or Rya-r44F^{d03686/k04913} mutants (Bonferroni’s, n.s.). Rya-r44F^{k04913/+} and Rya-r44F^{d03686/d03686} flies had significantly reduced ST50 values (Bonferroni’s, *p<0.05, **p<0.01). B. Genotype also significantly affected the development of rapid tolerance (one-way ANOVA, p<0.0001, n=20). Rya-r44F^{d03686/d03686} and Rya-r44F^{d03686/k04913} flies showed greatly blunted rapid tolerance to ethanol as compared to control (Bonferroni’s, *p<0.05, **p<0.01). All other groups did not differ significantly from one another (Bonferroni’s, n.s.).

Recapitulation of the abnormal phenotypes observed in the Rya-r44F transposon mutants was attempted by expressing RNAi against Rya-r44F. Expression of a UAS-RNAi transgene (Rya-r44F^{10963}) was driven in the nervous system (elav-Gal4, appl-Gal4,
repo-Gal4) or ubiquitously (da-Gal4) via the Gal4-UAS system. Considerable lethality was observed in elav-Gal4/Rya-r44F<sup>109631</sup> and da-Gal4/Rya-r44F<sup>109631</sup> crosses that prevented behavioral testing. Knockdown of Rya-r44F in neurons (appl-Gal4/Rya-r44F<sup>109631</sup>) and glia (repo-Gal4/Rya-r44F<sup>109631</sup>) resulted in neither lethality nor significant differences in sensitivity or rapid tolerance to ethanol (data not shown).

We attempted temporal induction of RNAi against Rya-r44F was through the use of an RU486 inducible Gal-4 system [96]. In addition, we fed flies the RyR antagonist dantrolene to pharmacologically inhibit RyR activity. Overall, these alternative approaches resulted in lethality or no significant change in ethanol sensitivity or tolerance (Table 2).

Table 2: Results of Attempts to Modify Rya-r44F Expression

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Intervention</th>
<th>Viability</th>
<th>Note</th>
</tr>
</thead>
<tbody>
<tr>
<td>da-Gal4;Rya-r44F&lt;sup&gt;109631&lt;/sup&gt;</td>
<td>n/a</td>
<td>Lethal</td>
<td></td>
</tr>
<tr>
<td>elav-Gal4;Rya-r44F&lt;sup&gt;109631&lt;/sup&gt;</td>
<td>n/a</td>
<td>Lethal</td>
<td></td>
</tr>
<tr>
<td>appl-Gal4/Rya-r44F&lt;sup&gt;109631&lt;/sup&gt;</td>
<td>n/a</td>
<td>Viable</td>
<td>No Δ ST50 / Tol.</td>
</tr>
<tr>
<td>repo-Gal4/Rya-r44F&lt;sup&gt;109631&lt;/sup&gt;</td>
<td>n/a</td>
<td>Viable</td>
<td>No Δ ST50 / Tol.</td>
</tr>
<tr>
<td>elav-Gal4-GS;Rya-r44F&lt;sup&gt;109631&lt;/sup&gt;</td>
<td>5d @ 5mM RU486 (Adult)</td>
<td>Viable</td>
<td>No Δ ST50 / Tol.</td>
</tr>
<tr>
<td>elav-Gal4-GS;Rya-r44F&lt;sup&gt;109631&lt;/sup&gt;</td>
<td>0.1-5mM RU486 (Dev.)</td>
<td>Viable</td>
<td>See Figure 17</td>
</tr>
<tr>
<td>tub-Gal4-GS;Rya-r44F&lt;sup&gt;109631&lt;/sup&gt;</td>
<td>0.1-5mM RU486 (Dev.)</td>
<td>Lethal</td>
<td></td>
</tr>
<tr>
<td>w&lt;sup&gt;118&lt;/sup&gt;</td>
<td>3d @ 10 mM Dantrolene</td>
<td>Viable</td>
<td>No Δ ST50</td>
</tr>
</tbody>
</table>

No Δ ST50 / Tol.: no significant change in SSA ST50 or rapid tolerance (data not shown). Adult - RNAi was induced in adult flies by feeding 5mM RU486 for 5 days. Dev - Crosses were performed on food pre-treated with various concentrations of RU486 to induce RNAi throughout development; flies were kept on RU486 treated food following eclosure.

Knockdown of Rya-r44F in the nervous system during development via RU486 inducible elav-Gal4-GS driver resulted in developmental delay but no lethality. elav-Gal4-GS;Rya-r44F<sup>109631</sup> flies exposed to 5mM RU486 during development exhibited significantly reduced ST50 values in SSAs as compared to vehicle treated flies of the same genotype (Figure 17A), while rapid tolerance remained unchanged (Figure 17B). However, these flies also displayed impaired basal locomotor function (Figure 17C), suggesting these results may be due to the effects of RU486 and not Rya-r44F.
knockdown, as we found that exposing flies to concentrations of RU486 ~5mM or higher causes increasing sensitivity to ethanol (Figure 18).

Figure 17: Effects of RU486 induced developmental expression of nervous system Rya-r44F RNAi on ethanol sensitivity and tolerance. elav-Gal4-GS/Rya-r44F^{10963} flies were grown on media supplemented with increasing concentrations of RU486 in order to induce expression of RNAi against Rya-r44F. A. Drug concentration had a significant overall effect on initial sensitivity to ethanol (one-way ANOVA, p=0.0083, n=4). Only flies reared on 5mM RU486 demonstrated altered ST50 values versus vehicle control (Bonferroni’s, *p<0.05). B. Rapid tolerance was not significantly affected by treatment with RU486 (one-way ANOVA, n.s, n=4). C. Baseline locomotor ability was assayed prior to the SSA. Concentration of RU485 had a significant effect on negative geotaxis (one-way ANOVA, p=0.0026, n=4), where 5.0mM led to a reduction in climbing activity (Bonferroni’s, **p<0.01).
Overall, the altered phenotypes of flies harboring mutant alleles of \textit{Rya-r44F} support a role for RyRs in ethanol-related behaviors in \textit{Drosophila}. These alleles did not produce dramatic changes in ethanol sensitivity and unexpectedly appeared to complement one another. However, rapid tolerance was greatly impaired in flies carrying any two lesions in the \textit{Rya-r44F} locus. These disparate results suggest these mutant alleles of \textit{Rya-r44F} induce complex changes in transcript abundance that is not reflective of a simple loss-of-function. Mechanistically, perhaps altered expression of ryanodine receptors in these mutants results in altered intracellular calcium homeostasis, and by extension, defects in the development of rapid tolerance to ethanol.

Unfortunately, these abnormal phenotypes could not be recapitulated by RNAi-mediated knockdown of \textit{Rya-r44F} due to the lethality observed in most genotypes and treatments. Attempting to use temporal knockdown of RyRs was also complicated by lethality and the toxicity of the agent of induction. In future studies, the temperature sensitive inhibitor of Gal4, Gal80$^\text{ts}$, may serve as a useful alternative to drug inducible expression systems for knockdown of \textit{Rya-r44F} \cite{97}. While dantrolene has been shown to antagonize invertebrate RyRs \textit{in vitro} \cite{98}, there are no reports of its efficacy when ingested. Thus, it is likely the extreme insolubility of dantrolene prevented its absorption \cite{99} in these experiments. In the future, substitution of dantrolene with the more soluble RyR antagonist azumolene \cite{100} or ryanodine may yield better results.
B. Loss of Clic Alters TGF-β Receptor Expression

**TGF-β Signaling in Drosophila**

The members of the transforming growth factor beta (TGF-β) superfamily of cytokines are critical for proper growth, cellular differentiation, and morphogenesis in eukaryotes. The seven transforming growth factor beta ligands in *Drosophila* are divided into the bone morphogenic protein (BMP) and Activin/TGF-β subfamilies. Mature ligands, formed by disulfide dimerization following precursor proteolysis, bind two types of membrane bound receptor serine/threonine kinases. These TGF-β receptors (TGF-βRs) exist as tetramers of two type I and II receptor subunits. The fly genome encodes three type I receptors, *thickviens* (*tkv*), *saxophone* (*sax*) and *baboon* (*babo*), and two type II receptors, *punt* (*put*) and *wishful thinking* (*wit*). Upon activation by ligand binding, the type II receptor subunits phosphorylate their neighboring type I receptor, which in turn leads to phosphorylation of intracellular R-SMADs. Activated R-SMADs translocate to the nucleus, where they elicit changes in gene transcription. Termination of the TGF-β signal cascade occurs via dephosphorylation of phospho-SMADs by protein phosphatases [101-103].

TGF-β signaling is critical for proper neurogenesis and is necessary for synapse formation at the *Drosophila* neuromuscular junction [104]. Additionally, the gene *BMPR1B*, a human ortholog of *tkv* and *babo*, has been associated with alcohol dependence [51]. In mammals the intracellular transducers of TGF-β signaling, Smad2 and Smad3, interact with a complex of CLIC4 and Schnurri-2 to facilitate nuclear translocation following activation [90]. In the nucleus, CLIC4 protects p-Smad2 and p-Smad3 from dephosphorylation, thereby amplifying TGF-β signaling [90]. Taken together, these findings suggest that the TGF-β pathway may be involved in behavioral responses to ethanol. Considering the ability of CLICs to potentiate the TGF-β pathway, we
predicted that that loss of Clic function in Drosophila would alter expression of the type II receptor encoded by tkv.

Results and Discussion

Flies containing the strong loss of function Clic<sup>G0472</sup> and Clic<sup>EY04209</sup> alleles were bred to homozygosity, and expression of Clic and tkv were determined by qRT-PCR. As compared to a w<sup>1118</sup> control, Clic expression was virtually undetectable in Clic<sup>G0472/G0472</sup> and Clic<sup>EY04209/EY04209</sup> mutants. Expression of tkv was elevated in both mutant genotypes with a greater than two-fold increase of tkv detected in Clic<sup>G0472/G0472</sup> flies (Figure 19).

![Figure 19: Increased tkv expression coincides with loss of Clic function.](image)

Whole body Clic and tkv mRNA expression as determined by qRT-PCR in flies homozygous for G0472 and EY04209 transposons relative to w<sup>1118</sup> controls. tkv expression was significantly elevated in Clic<sup>G0472/G0472</sup> flies (one sample t-test, **p<0.01, n=3).
The increased expression of tkv in flies deficient in Clic may be an adaptive response. As CLICs have stabilizing effects on p-SMADs, loss of Clic function could possibly aberrantly attenuate the TGF-β signaling pathway. Upregulation of TGF-β receptors, specifically tkv, could reflect a mechanism that restores normal TGF-β signaling through greater receptor kinase capacity. Expression levels of other genes encoding TGF-β subunits (e.g. wit, put, babo) remain to be determined. Genetic manipulation of the genes for the TGF-β receptors appears to affect sensitivity to ethanol (Figure 1 and data not shown), but remains inconclusive at this time.

Determining the influence of the TGF-β pathway on ethanol-related behaviors may be difficult by genetically ablating any one single TGF-β receptor subunit. Targeting the downstream transducers of the pathway, such as SMADs, may result in more potent interruption of TGF-β signaling and altered responses to alcohol. Alternatively, TGF-β receptor kinase inhibitors could be used for pharmacological manipulation of the TGF-β pathway.

D. Clic Network Analysis & Screening

Multi-Species Gene Networks

The rapid expansion of high-throughput sequencing technologies and bioinformatics has allowed for the construction of large multi-species genomic and transcriptomic databases. By exploiting this wealth of information, it is possible to construct experimentally evidenced networks of genes associated with particular phenotypes or diseases. By integrating data from multiple species into these networks, highly refined and target rich sets of candidate genes can be obtained. Necessarily comprised of genes with strongly conserved biological functions, experimental interrogation of these networks can be readily performed in multiple animal models. As a
result, such studies provide opportunities for generating powerful and immediately relevant insight into complex biological questions.

Due to the large number of genes in these networks and temporal limitations, comprehensive screening of network members can be accomplished only in invertebrate models. By examination of one such network, nucleated on murine Clic4 and fly Clic, we have identified many genes as novel regulators of ethanol-related behaviors.

Results & Discussion

We compiled and queried gene expression data from both public and private datasets (courtesy of Michael Miles) for genes co-regulated with Clic4 by ABBA (Anchored bicliques of biomolecular associates) analysis in GeneWeaver [105]. A list of 157 murine genes was obtained and converted, where possible, into orthologs in flies (144), worms (117) and humans (150) with g:Profiler [106] and NCBI Homologene. Using these lists, a network of genes predicted to interact with those on our input lists was constructed using the GeneMania tool [107] and the gene ontology functions in FlyBase [71]. The result of this work was a refined network composed of 140 genes predicted to interact with Clic directly or indirectly via shared pathway. This network contains many genes or members of gene families known to influence ethanol-related behaviors in flies (Clic [64], aru [52], Pka-RII [24]) and genes that have altered expression in human alcoholic brain (14-3-3ε and 14-3-3ζ [105]). The network is also over-represented for genes involved in vesicle trafficking (p=3.5×10^{-2}) and G-protein signaling (1×10^{-2}), suggesting that the network may influence ethanol-related behaviors via these processes.

Next, flies expressing RNAi against one of each network member were tested in SSAs. Knockdown of gene network members resulted in significant changes in ethanol sensitivity or rapid tolerance in 20 of 67 (~30%) of those tested. Interesting hits from this
screen represent genes involved in calcium binding, spliceosome regulation, electrical synapsis, cAMP signaling, neurotransmission, and fatty acid metabolism (Figure 20).

Excitingly, many of the genes identified by this initial Clic network analysis have not been previously implicated in regulating ethanol related behaviors. Further validation of these findings will require expanded behavioral testing (of both flies expressing RNAi and transposon insertion mutants) and verification of target gene knockdown. Additionally, follow-up studies in nematodes and rodents will help clarify the relevance of promising candidate genes in human alcohol abuse and tolerance.

**Figure 20:** Changes in sensitivity and rapid tolerance to ethanol in flies expressing RNAi against Clic network members. A. Lines expressing RNAi against indicated gene with a greater than 20 percent change in ethanol sensitivity in SSAs versus elav-Gal4/+ control. B. Lines expressing RNAi against indicated gene with a greater than 20 percent change in rapid ethanol tolerance in SSAs versus elav-Gal4/+ control.
E. Conclusions

Noting the conserved role of CLICs in regulating behavioral responsive behaviors, we sought to investigate the influence of genes associated with Clic on these behaviors in flies.

We found that flies containing lesions in Rya-r44F, the gene encoding ryanodine receptors, are defective in their ability to develop rapid tolerance to ethanol. Unfortunately, further attempts to augment RyR expression or function were inconclusive. The preliminary studies that we performed on the TGF-β receptors were similarly vague. However, contrary to being exclusionary, our results sustain probable roles for ryanodine receptors and the TGF-β signaling pathway in ethanol-related behaviors. Elucidating the mechanisms and the extent in which these pathways influence response to alcohol will require a great deal of additional work. Importantly, these future studies will require a multi-gene or systematic approach.

In a separate line of investigation we developed one such systematic approach through the construction and analysis of a multi-species gene network. Using gene expression data from different model organisms we constructed a network of genes predicted to interact with Clic. Subsequent behavioral testing of flies expressing RNAi versus these network members revealed many new potential modulators of ethanol sensitivity and tolerance. Based on the encouraging outcome of this pilot network analysis, multi-species gene networks anchored around Rya-r44F, GSK3β, and the TGF-βR superfamily of genes have begun to be examined by members of the Grotewiel lab. In summary, these network-based behavioral genetic screens hold great potential for identifying new translational targets for the prevention and treatment of alcohol use disorders.
Materials & Methods

Fly Stocks and 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 unless otherwise stated. The \( w^{1118} \) control strain used in these studies (a.k.a. \( w[A] \)) is isogenic for the \( X, 2 \) and \( 3 \) chromosomes was obtained from the Drosophila Stock Center (stock# 5905, Bloomington, IN, U.S.A.). The elav-Gal4 driver, Clic (G0472), 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 (\( w[VDRC] \)), two UAS-white-RNAi strains (\( w^{30033} \) and \( w^{30034} \)) and a UAS-Rya-r44F-RNAi (\( Rya-r44F^{109631} \)) strain were obtained from the Vienna Drosophila RNAi Center (Vienna, Austria). A chromosome harboring both the elav-Gal4 driver and the \( w^{30034} \) RNAi transgene (\( elav-Gal4, w^{30034} \)) was generated via meiotic recombination and then balanced over FM7i. A stock homozygous for a wild-type \( w \) allele in the \( w[A] \) background was generated by backcrossing a Canton-S \( X \) chromosome to \( w[A] \) for 7 generations.

eRING assays: Analysis of ethanol sensitivity in eRING studies were performed as previously described [25] using 20-50% ethanol.
Eye imaging: Digital photographs of adult eyes were obtained using a QImaging Micropublisher 3.3 camera with QCapture 2.9.12 software (QImaging, Surrey, BC Canada) and a Nikon SMZ-2T microscope (Nikon Instruments Inc., Melville, NY, U.S.A.).

Simple Sedation Assay (SSA) for ethanol sensitivity and rapid tolerance: Adult flies were grown as above, immobilized under light CO$_2$ anesthesia, separated by sex and genotype, and then placed into fresh food vials (11 flies/vial) to recover from anesthesia overnight at 25°C/60% relative humidity prior to any behavioral tests. Adult flies (2-5 days-old) were used for all experiments and all comparisons between groups were based on flies that were grown, handled and tested side-by-side. Each vial of 11 flies represents an n=1. All SSAs were performed at 23-25°C and 50-55% relative humidity under standard laboratory lighting. The experimenter was blind to genotype in all studies. SSAs were initiated by transferring adult flies into empty 2.5 x 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 S2). The number of dead/inactive flies (on average less than 1%) was recorded for each vial (t=0). Starting with the first row of 4 vials, 2 ml of ethanol (0-50%) 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, each vial was gently tapped 3 times on a table and the number of sedated flies (i.e. flies unable to right themselves and/or move from the floor of the vial) in each vial was recorded 30 seconds after the final tap. SSAs were terminated typically at 60-90 minutes or when all flies were fully sedated. 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) values were interpolated from third-order polynomial curve fits (the least complex curve that fit the data well, \( R^2 = 0.96\pm0.001, n=1221 \)) using Excel (Microsoft, Redwood, WA) or Prism 4.03 (GraphPad, San Diego, CA) from the time-course data for each vial. Rapid tolerance was determined in SSAs 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 SSAs. The development of rapid tolerance to ethanol was expressed as a ratio between the ST50_{EE} and ST50_{E} as similarly reported [12, 26, 35, 36].

**Weight-loss during SSAs:** Groups of 10 flies were weighed before and after exposure to vapor from water (mock) or 50% ethanol in SSAs.

**Internal ethanol:** Internal ethanol was determined as previously described [25] in flies exposed to vapor from various concentrations of ethanol (0-50%) in SSAs. Exposure time is indicated in the figure legend for each experiment. Data are presented as mean internal ethanol concentration minus average baseline (0 min) ethanol content.

**Locomotor assay:** A negative geotaxis assay was adapted from Rothenfluh et al 2006. Ten flies were collected into food vials, allowed to recover overnight, and transferred into an empty 19 cm tall tube composed of two food vials secured end-on-end (NG vial). Each NG vial was arranged in a grid pattern as described for SSA. At 15 second intervals, each NG vial was gently tapped and the number of flies that climbed at least half the height of the NG vial (9.5 cm) within 15 seconds was recorded. This was repeated three times for each NG vial and averaged for n=1 per NG vial. A negative geotaxis index was calculated from each NG vial by dividing the average number of flies
that reach the half-way point by the total number of flies. A mock-SSA (using 0% ethanol) was then performed on each group of flies by quickly converting each NG-vial into a SSA setup as described above. Following the mock-SSA, negative geotaxis was again assessed by converting each SSA vial back into an NG vial.

**qRT-PCR:** Clic and tkv expression was determined by qRT-PCR using standard methods as previously described [108].

**Drug Administration:** RU486 (Sigma Chemical Co., St. Louis, MO, USA) was diluted in 100% ethanol to the desired concentration. 100uL of each RU486 solution was aliquoted onto fresh food vials and allowed to dry overnight prior to the introduction of adult flies. Developmental exposure to RU486 was accomplished by pre-treating fresh food vials with 500uL of RU486 solution prior to seeding by parental flies. Adults arising from these crosses were transferred into fresh food vials pre-treated with RU486. Dantrolene sodium salt (Sigma Chemical Co., St. Louis, MO, USA) was dissolved in 50% methanol 50% DMSO to produce stock solutions of the desired concentrations. Dantrolene was delivered by pre-treating food vials (as above) or diluted in liquid food by capillary tube as previously described [109].

**Statistical Analyses:** Goodness of fit for first-, second-, third- and fourth-order polynomials on SSA time-course data were compared using F tests (Prism, GraphPad Software, San Diego, CA). Correlations, t-tests, and ANOVAs with post-hoc multiple comparison tests were performed in Prism 4.03 (GraphPad Software, San Diego, CA). p<0.05 was considered statistically significant. Data are presented as means ± SEM.
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References


Appendix

eRING Protocol

I. Fly Collection: day before eRING testing
   1. 12-16 days after bottles are seeded with adults, there should be enough new flies (i.e. progeny) for eRING tests.
   2. On the day before eRING testing, place in the environmental chamber 5 non-yeasted food vials for each group or genotype to be tested. Allow to warm to room temperature. Vials must have NO condensation on sides.
   3. Anesthetize flies to be tested. Collect groups of 25 flies of appropriate sex using minimal CO\(_2\) anesthesia into a 1.5 mL tube and then dump the flies from the 1.5 mL tube into a non-yeasted food vial (warm and dry as in 2). Label the vial accordingly and place the vial on its side until the flies are awake.
   4. Place the non-yeasted food vials with flies in the environmental chamber overnight to recover from the anesthesia.

II. Preparation of Flugged Vials: day before eRING testing
   1. Prepare 1 flugged vial for each vial of flies collected above.
   2. Flugs are in the fly room on the top shelf of the middle bench on the side facing the dissecting microscopes.
   3. Cut Flugs with a razor blade (be careful) into 3 same-sized portions.
   4. Place glue on one side of the flug. Add just enough so that the glue does not drip off the flug, and try to keep the glue to the center of the flug. This will help prevent glue from getting on the sides of the vials when the flugs are added. Glue used for this procedure is found in the top drawer in the middle of the bench with the dissecting scopes (Tombo brand Aqua Liquid Glue).
   5. Place the flug with the glue into an empty, clean vial (i.e. without food) using forceps. Gently guide the flug to the bottom of the vial so that glue does not get on the sides of the vial. At this point the flug is pointing vertically. When you do get it so that it is touching the bottom of the vial, gently push it so that the side with the glue is facing the bottom of the vial. Using the blunt end of forceps, smash the flug into the bottom of the vial so that it fits snuggly and will not come loose.
   6. Repeat this for all vials needed for the experiment. Once the flugs are glued, turn vials upside down in a vial box and place them either on the bench in the fly room or in the RING testing room. Allow to dry overnight.

III. eRING Testing
   A. Preparation
      1. Turn on humidifier in testing room to bring humidity to 55-60%. Make sure there is water in the humidifier reservoirs.
      2. Dilute ethanol with ddH\(_2\)O from the purifier. You will need 500 µl for each ethanol vial. **ALLOW ETHANOL SOLUTION TO RETURN TO ROOM TEMP!**
      3. Fill an additional bottle/tube of ddH\(_2\)O. You will need 500 µl for each water vial.
      4. Turn on the light sources on the eRING table.
      5. Place memory card in camera. Align camera so that it frames the table and produces a square picture. Take a test picture before the experiment to ensure correct alignment.
      6. Bring the flies into the test room to acclimate at least 30 minutes before the test begins.
B. Testing: You will assess negative geotaxis using TWO protocols for each group of flies. Each group of flies will be tested three times for BASELINE CLIMBING in the presence of water. Each group of flies will then be tested for TIME-DEPENDENT CLIMBING in the presence of water or ethanol. These two protocols are integrated such that preparation for the second begins right before the first is completed.

- **Baseline Climbing**
  1. Prepare one rig for baseline climbing and another for time-dependent climbing. Use tape to label each on the right panel of the rigs describing the genotype, the test (i.e. water or ethanol) and date.
  2. Secure flugs to five vials by pressing down on the flugs with the blunt end of forceps.
  3. Pipette 500 µL of ddH₂O onto the flug of each vial being careful not to drip water onto the side of the vial. These are now water vials.
  4. Transfer 5 sets of 25 flies into individual water vials using a funnel. Steady the funnel so as not to crush flies against the side of the vial. After transferring flies, cover each vial quickly with an orange cap so that no flies escape. Place each vial with flies and cap into rig.
  5. Carefully place the top on the rig so that the orange caps and vials fit securely.
  6. Place the rig in front of the light diffuser and make sure the camera is turned on.
  7. Start the timer and immediately rap the rig three times on the table in the first 2 seconds.
  8. When the timer reaches 6 seconds, press the shutter button on the camera to take the picture. This will allow the flies to climb for 4 seconds. It will help to prime the camera by pressing the shutter button halfway down at 5 seconds to avoid any shutter delay when the button is pressed fully at 6 seconds.
  9. Wait until the timer reaches 60 seconds and rap the rig against the table 3 times in 2 seconds.
  10. When the timer reaches 1 minute 6 seconds, take a second picture as in step 8 above.
  11. During the interval before the next water test (you will have ~50 seconds), pipette 500 µL of EtOH into each ethanol vial. Keep vials in cardboard grid and cover with orange caps. These are now ethanol vials.
  12. When the timer reaches 2 minutes, rap the water rig 3 times in 2 seconds and take a third water picture at 2 minutes 6 seconds as in step 8 above. Testing of baseline climbing is now complete.

- **Time-Dependent Climbing**
  13. Immediately remove lid from water rig and transfer flies from water vials to ethanol vials prepared between second and third baseline climbing tests. Use a funnel for the transfer. Steady the funnel so as not to crush flies against the side of the vial. Cover each vial quickly with an orange cap so that no flies escape. Place each vial and cap into ethanol rig after each transfer of flies. Do not stop timer. The transfer must be completed within 2 minutes (when the timer reaches 4 minutes).
  14. Carefully place the top of the rig so that the vials and orange lids fit securely.
  15. Place the rig in front of the light diffuser and make sure the camera is turned on.
  16. When the timer is at 4 minutes, reset it to zero and restart the timer.
  17. Immediately rap the rig three times on the table in the first 2 seconds.
  18. When the timer reaches 6 seconds, press the shutter button on the camera to take the picture. This will allow the flies to climb for 4 seconds. It will help to
prime the camera by pressing the shutter button halfway down at 5 seconds to avoid any shutter delay when the button is pressed fully at 6 seconds.
19. Repeat steps 17 and 18 at 1 minute intervals, continuing out to 19 minutes (20 tests) or until determined by the experimental design.
20. Flip flies back into their vials. At this point the testing is done for this set of flies. You may either throw out these flies or keep them for other types of testing.
21. Repeat the above steps for each set of flies being tested.

NOTE: Record the pictures being taken in a notebook for reference when doing data analysis.

• Clean-up
  1. Remove flugs from vials and discard flugs. Place vials and orange caps into a gray bin beside the sink in the fly room to be cleaned.
  2. Turn off light source, camera and humidifier
  3. Make sure rig is put away and everything is in order
  4. Turn off light and close and lock door to testing room when done.

C. Data Analysis
• Primary Data Analysis
  1. Put memory card into card reader and plug card reader into the computer. Copy pictures from card into a folder on your desktop.
  2. Open Adobe Photoshop 5.0
  3. In Photoshop open pictures you wish to edit. (NOTE: Photoshop has an upper limit of the number of pictures that can be opened at a time. 18 pictures is usually a good number to work with at a time)
  4. On the right hand side of the screen a menu should be present in the menu press the “Create New Action” button. This will cause a screen to appear asking you to name and change certain settings. You can name the action if you wish otherwise press “Record” to continue.
  5. Use the marquee tool to draw an outline around the vials and the panel with the label. In the Image menu in the toolbar select crop.
  6. In the Image menu in the toolbar select Mode, then select grayscale. It will ask if you wish to discard color information, press ok to continue.
  7. In the Image menu in the toolbar select Image Size. This will come up with a screen where you can adjust the picture. Change image height to 3.25 inches. This will automatically adjust the width. Press ok to continue.
  8. In the File menu in the toolbar select Save As. Save a copy of the picture in TIFF format to the folder you created on your desktop.
  9. Close the picture that was being edited.
10. Press the stop button on the right hand menu to stop the recording
11. Press play for each picture, this will do all of the above steps in editing the picture.
12. Continue for all the pictures for the experiment.
13. Close Photoshop and open Scion image.
14. In Scion Image open options in the Analyze menu in the toolbar. Make sure that only X&Y Center is checked, the max measurement is set to 8000, the field width is 9 and the digits to the right of decimal point are 5. In the Options menu select preferences and make sure the Clipboard Buffer Size is set 99999 and the boxes for Invert Y-axis and Desktop Friendly are checked. If these are not the defaults then change them, and in the file menu select record preferences to make these the default choices.
15. In the toolbar open the Special Menu. In the Special menu select Load Macros.
16. In the Macros folder select the ScreenMacros
17. Open TIFF formatted pictures in the file menu (NOTE: It might be easier to start off with only 3 or 4 pictures)
18. Once the pictures are open, select Subtract Background from the Special Menu
19. Select Threshold in the Special Menu. All pictures that are opened in Scion Image will be edited when Subtract Background and Threshold are executed. It is therefore unnecessary to repeat this process for each picture open.
20. Choose the eraser tool and erase anything in the picture that is not a fly.
21. Choose the pencil tool and make the “ink” white. Draw lines to separate any flies that look to be joined in the picture.
22. Photo analysis in Scion Image:
   a. When analyzing the three pictures taken in the Baseline Climbing test, edit each picture then select Analyze Particles from the Special Menu. This will analyze the particle of any picture that is open so it is important to have only the water pictures of a single genotype open at this time and that the pictures have been edited as described in steps 18-21. Scion Image will create a two column sheet that opens in an individual window. This sheet has the measurements for all three water pictures. Copy and paste these data points into the W 1 2 3 tab in columns A and B in the excel sheet “eRING Master Neg Geo Perf Frac Half Max AUC 2012.04”.
   b. For the pictures taken during the Time-Dependent Climbing test, individually analyze them by selecting Analyze Particles in the Analyze menu. In the screen that pops up make sure minimum particle size is set to 3 and max is set to 50. Make sure the options for Label Particles and Reset Measurement Counter are selected only. The picture will now have numbers where the flies are. Click on the picture and press Ctrl-C to copy these values. Paste the values into the corresponding time point (E1= picture of 1st test) in the same excel sheet as in step 22a. Repeat this for each picture in the rig until all pictures for Time-Dependent Climbing test have been analyzed.
23. Once all of the data from Scion image has been compiled into the spread sheet, press ‘Ctrl E’ to compile the data.

● Secondary Data Analysis: T50 values
   1. Copy compiled ethanol data from green cells in NG and PF sheets into separate X-Y single replicate data sheets in Prism.
   2. For each set of data, in Prism, click Analyze, Curves and Regression, Select data sets (if necessary), Click OK. Select Third Order Polynomial, select Unknowns from Standard Curve, Click OK.
   3. T50 values are found in the first column in Interpolated X values.
   4. T50 values are calculated for the data in both the Neg Geo and Perf Frac sheets.

● Secondary Data Analysis: Percent Area Under the Curve
   1. Open the completed eRING master file.
   2. In the Neg Geo sheet, copy the blue cells from the Total Area Under Curve table.
   3. Open the eRING % AUC worksheet.
   4. Paste total AUC values into the blue cells labeled Total AUC in either the H2O Vials - Negative Geotaxis table or the EtOH Vials – Negative Geotaxis table, as appropriate.
5. The green cells labeled % AUC will return the percent area under the curve values.
6. Repeat steps 2-5 using the blue cell values from the Perf Frac sheet in the eRING master file and paste into the Performance Fraction tables in the eRING % AUC worksheet.

D. Camera Settings

Menu:

1st Tab (Camera)
- Flash Syn: 1st Curtain
- Slow Synchro: Off
- Red-eye: Off
- Cont. Shooting: Picture multiple boxes
- Self-timer: 2
- Wireless Delay: 0 sec
- Spot AE Point: Center
- ND Filter: Off
- MF Point Zoom: On
- AF Mode: Single
- AF assist Beam: Off
- Digital Zoom: Off
- Review: 4 sec

2nd Tab (Wrench and Hammer)
- Beep: On
- LCD Brightness: Picture Bright Sun
- Auto Power: On
- Date/Time: Adjust if not correct
- Format: Gray on old camera, 1st setting on new camera
- Volume: Center setting
- File # Reset: Off
- Auto rotate: On
- Distance Units: m/cm
- Language: English
- Video System: NTSC
- Communication: Normal

3rd Tab (Person and camera): All setting should be on 1.

Camera dial should be set to P mode.

Function Menu:
- ISO Speed: 100
- 1st Off tab: Effect Off
- 2nd Off Tab: BKT-Off
- +/- Tab: +/- Flash Set to 0
- M2: M2
- Shutter Speed: Old Camera: 1/240, new Camera: 1/50
Simple Sedation Assay Protocol

A. Day before assay

1. Collect flies (reared for behavioral assays) in groups of 11 (single sex) under brief CO₂ following standard procedures for behavioral assays.

2. Allow flies to recover overnight in non-yeasted food vials in the environmental chamber. It is possible to test a maximum of 24 vials of flies in a single experiment.

3. Prepare ethanol solutions – allow to return to room temperature in testing room overnight

B. Day of assay

1. For each vial of flies to be tested, you will need (a) a clean, empty food vial; i.e. testing vial, (b) a new unmodified flug, (c) a silicone or rubber #4 plug and (d) 2 ml of ethanol solution.

2. Turn on humidifier and allow relative humidity in testing room to rise to 55-65%.

3. It is important to be blinded to genotype during testing. Assign a unique code to each group of vials for each genotype and record the code for later. Alternatively, arrange vials in a randomly staggered order so that each genotype is tested at each position in the testing queue and record this order for later review. It is possible to test sets of 4 vials simultaneously, so arrange 24 vials (maximum) in 5 sets or rows containing 4 vials each. Place coded vials with flies in testing room to acclimate.

3. Label empty testing vials #1-24 with marker or use codified labels.

4. Print a copy of a testing log from the SSA Excel worksheet and by enter the code for each vial into the Test Log. Add other pertinent information (% ethanol, sex, etc.) to the Test Log worksheet.

5. Transfer all flies from all food vials into matched/labeled testing vials one at a time and immediately insert flugs into testing vials until flugs are slightly below the vial tops. Use fluginator to push flugs down into vials. It may help to first ease the flug into the vial with your thumb.

6. Time 0 assessment: For each vial individually, grasp with thumb and forefinger, tap gently on the table three times to knock flies to the bottom of the vial, wait 30 seconds and then count the number of flies that are immobile. Typically, this is 0 or 1. Record the number of immobile flies for each vial at time 0 in the printed Testing Log.

7. Hereafter, each row of four vials will be handled as a set at staggered one-minute intervals.

Start timer counting up at time 0 and immediately begin adding 2 ml of ethanol to the flug in the vials for the first row/set of 4 vials. Add ethanol to the vials at 5 second intervals in the order they will be tested. Add ethanol in a circular motion so that all ethanol is
absorbed in the flug. When ethanol has been added to all four testing vials in the set, insert a rubber or silicone #4 plug in each vial to seal it.

At times 1, 2, 3, 4, and 5 minutes, add 2 ml of ethanol to the second, third, fourth, fifth, and sixth sets of four vials, respectively. Continue inserting #4 stoppers after adding ethanol.

9. At time 6 minutes, test the first set of 4 vials by grasping each vial with thumb and forefinger, tapping gently on the table three times to knock flies to the bottom of the vial, waiting 30 seconds and then counting and recording the total number of flies that are sedated. Flies are scored as sedated when they are unable to flip off their backs, or lying upright with legs spread apart – often when their heads resting against the bottom of the vial.

Handle each vial within the set at 5 second intervals. Specifically, vial one is tapped at 6 minutes 0 seconds and assessed at 6 minutes 30 seconds. Vial two is tapped at 6 minutes 5 seconds and assessed at 6 minutes 35 seconds. Vial three is tapped at 6 minutes 10 seconds and assessed at 6 minutes 40 seconds. Vial four is tapped at 6 minutes 15 seconds and assessed at 6 minutes 45 seconds.

At times 7, 8, 9, 10, and 11 minutes, test the second, third, fourth, fifth, and sixth sets of vials, respectively, as done for the first set.

10. At time 12 minutes, test the first set of 4 vials again as described in B9 and continue testing the second, third, fourth, fifth, and sixth sets of vials at 13, 14, 15, 16 and 17 minutes, respectively.

Continue testing flies as described in B9 and B10 out to 60 minutes or until all flies are sedated.

Clean-up is (a) turn off humidifier, (b) remove #4 plugs for later reuse, (c) testing vials (flug, tube, & flies) in trash, (d) remove any trash from and straighten up testing room and (g) turn off light in testing room.

**IF ASSESSING RAPID TOLERANCE** - Return sedated flies to their original food vials after the SSA run and place in environmental chamber for 4 hours. After recovery, flies can be re-tested in a SSA by returning to step B1. Allot up to 120 minutes of testing time for the second exposure as control flies will develop rapid tolerance to ethanol.

11. Enter the total number of flies in each vial in the Test Log within the Excel SSA worksheet file. Enter the number of sedated flies for each vial at each time point as recorded on the printed sheet (and second exposure data if applicable). Percent Active flies will be automatically calculated and graphed below the Test Log. Press ‘Ctrl + e’ to calculate ST50s (and ST50 EE/E if applicable) and sort data by genotype.
Basic Locomotor Assay Protocol

A. Day before assay

1. Collect flies (reared for behavioral assays) in groups of 10 (single sex) under brief CO₂ following standard procedures for behavioral assays.

2. Allow flies to recover overnight in non-yeasted food vials in the environmental chamber. It is possible to test a maximum of 24 vials of flies in a single experiment.

B. Day of testing

1. For each vial of flies to be tested, you will need:
   - (5) clean unused food vials
   - (1) clean flug
   - (1) clean silicone stopper
   - 1mL of water
   - Clear tape

2. Turn on humidifier and allow relative humidity in testing room to rise to 55-65%.

3. It is important to be blinded to genotype during testing. Assign a unique code to each group of vials for each genotype and record the code for later. Alternatively, arrange vials in a randomly staggered order so that each genotype is tested at each position in the testing queue and record this order for later review. It is possible to test sets of 4 vials simultaneously, so arrange 24 vials (maximum) in 5 sets or rows containing 4 vials each. Place coded vials with flies in testing room to acclimate.

3. Label 5 empty testing vials #1-24 with marker or use codified labels. **Wipe all test vials with a damp Kim-Wipe to dissipate any static charge.**

4. Print a copy of a testing log from the Locomotor Excel worksheet and by enter the code for each vial into the Test Log. Add other pertinent information (% ethanol, sex, etc.) to the Test Log worksheet.

5. Transfer all flies from all food vials into a matched/labeled testing vial one at a time. Immediately place another clean vial end-on-end with the vial containing flies and tape in place. The result will be a conjoined tube 19cm in height (NG TUBE) containing flies.

6. You will now take three measurements of negative geotaxis for all NG tubes. At time 0 minutes, test the first row of 4 NG tubes. For tube $n$:

   - Grasp each vial with thumb and forefinger & tapping gently on the table three times to knock flies to the bottom of the vial.
   - Over the next 15 seconds count and record the number of flies in the NG tube that climb or fly above the half-way mark (9.5cm). Be careful not to count flies that cross into the top and later into the bottom of the tube twice.
   - At the end of this 15 second period tap then next tube ($n+1$) and score over the next 15 second.
   - This cycle repeats until each vial has been scored at least three times.
**It is important to maintain an offset of ~6 min between re-testing of the same vial – if running less than 24 NG tubes offset the restarting of the second and third testing cycles accordingly**

7. Following this first negative geotaxis assay, snap off the top half of each NG tube and seal with a clean flug – make sure no flies escape. Essentially, the NG tube has now been converted into an SSA tube. Perform a mock SSA run with these flies – picking up from step B5 of the SSA protocol – substituting 1mL of water in place of 50% ethanol. Do not record # of sedated flies unless specifically desired.

8. Immediately following cessation of the 1 hour mock-SSA run, transfer flies into a third set of clean matched food vials. It is often helpful to seal the mock-SSA tubes with a cotton ball after removal of the wet flugs – this will absorb excess fluid and keep flies from escaping. The goal here is to put the flies in a new NG tubes as in step B5 (above).


10. Clean-up; used plastic tubes do not need to be kept.

11. Enter raw climbing data in the Locomotor spreadsheet – it will automatically average the three performance measurements of each NG tube – this average will be converted into an NG ratio.

12. These data can now be plotted and analyzed in Prism.
Ethanol Content Protocol

**Use pre-chilled solutions throughout assay for consistency**

1. Grow/collection flies and prepare Flugged vials as you normally would for eRING assays. Use 25 flies/vial and typically 1 vial/genotype with 3-5 vials total per test. Expose one group to ddH₂O (0 minutes) and other groups to ethanol (0.5 ml in Flug) during eRING tests for 5 and 10 minutes or other times as appropriate.

2. After each water or ethanol exposure, transfer flies to labeled 1.5 ml snap-cap tubes and store at -20°C. Continue water and ethanol exposures until you have a complete set of frozen flies from each genotype at each time-point.

3. Homogenize frozen flies with drill/pestle in 200 μl ice-cold ddH₂O for 30 seconds. Keep flies on ice before and after homogenization.

4. Centrifuge homogenized flies at maximum speed at 4°C for 20 minutes. Prepare 25 mM standard by adding 4.37 μl of 100% ethanol to 2996 μl ice-cold ddH₂O. Prepare remaining standards using the table below. Store standards on ice.

5. Transfer 100 μl of clear supernatant to new labeled 1.5 ml snap-cap tube. Lipid or other crud will stick to the outside of the pipette tip. DO NOT TOUCH PIPPETTE TIP TO NEW TUBE! Store 100 μl supernatants on ice.

6. Add 300 μl of cold ethanol reagent to 1.5 ml snap-cap tubes for each sample (in triplicate, 3 tubes/sample) and standard (in duplicate, 2 tubes/standard).

7. Add 3 μl of each sample supernatant and standard to the corresponding tube from step 6. Mix by single pulse vortexing.

8. Incubate at 30°C in heat block for 5 minutes.

9. Read absorbance of 100 μl of each reaction at 340 nm and interpolate values for samples in Prism.

10. Final ethanol concentration in samples determined as: mM interpolation x (250 μl/[# flies x μl/fly]). Fly volume in μl is determined as indicated on next page. Each vial of flies is an N of one.

Notes:

1. Standards

<table>
<thead>
<tr>
<th>Standard</th>
<th>Volume of ddH₂O</th>
<th>Volume of 25 mM</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 mM</td>
<td>1000 μl</td>
<td>0</td>
</tr>
<tr>
<td>5 mM</td>
<td>800 μl</td>
<td>200 μl</td>
</tr>
<tr>
<td>10 mM</td>
<td>600 μl</td>
<td>400 μl</td>
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<tr>
<td>15 mM</td>
<td>400 μl</td>
<td>600 μl</td>
</tr>
<tr>
<td>20 mM</td>
<td>200 μl</td>
<td>800 μl</td>
</tr>
<tr>
<td>25 mM</td>
<td>2996 μl</td>
<td>4.37 μl (100% ethanol, 17.16 M)</td>
</tr>
</tbody>
</table>
2. Alcohol Reagent: Dilute per manufacturer’s instructions. Good for at least 2 weeks at 4°C.

3. Reaction is maximal at ~2 minutes and has a stable product (i.e. A340) out to at least 12 minutes.

4. Use all cold reagents for consistency.

5. A 10 minute exposure to vapor from 50% ethanol in an eRING assay should lead to a final internal ethanol concentration of ~150 mM in control flies. Each vial of flies is an N of one.
Fly Volume Protocol

1. Grow/collect flies as you normally would for eRING assays. Use 25 flies/vial and typically 3 vials/genotype. Each vial of flies is an N of one.

2. Drill 2-3 holes in the lids of 1.5 ml snap-cap tubes (one tube for each vial of flies in step 1) using a flame-heated needle (~20 gauge, large enough for CO$_2$ to enter but small enough to retain flies). Be careful!

3. Weigh each 1.5 ml snap-cap tube from step 2 using the Mettler PB153S balance and record the tube weight out to 3 decimal places on the side of each tube or elsewhere. This is the *tube weight*.

4. Anesthetize flies in vials and transfer to weighed tubes from step 3. Place tubes upside down on CO$_2$ plate in quarantine area to keep the flies anesthetized.

5. Weigh each tube containing flies from step 4 using the same balance and record the weight out to 3 decimal places on the side of each tube or elsewhere. This is the *tube wet weight*.

6. Place the tubes with flies in a 55°C dry incubator for 18-24 hours to desiccate.

7. Weigh each tube containing desiccated flies from step 6 using the same balance and record the weight out to 3 decimal places on the side of each tube or elsewhere. This is the *tube dry weight*.

8. Determine the total weight of each fly: ([tube wet weight] – [tube weight])/# flies. This should be ~1.5 mg/fly. Females will be bigger than males.

9. Determine the water weight of each fly: ([tube wet weight] – [tube dry weight])/# flies. This should be ~0.8 mg/female and 0.6 mg/male.

10. One mg of water weight = one µl of water volume. The water volume is used to calculate the internal ethanol concentration (see previous page).