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The Role Of Dunc13 In Ethanol Tolerance

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THE ROLE OF DUNC13 IN ETHANOL TOLERANCE

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ABSTRACT

Although the effects of ethanol on presynaptic activity have been studied, the molecular mechanisms and the changes in gene expression which are responsible for inducing ethanol tolerance are unclear. Munc13-1 is an active-zone protein that is essential for presynaptic vesicle fusion. This protein interacts with vesicle fusion machinery at presynaptic active zones in the mammalian brain. The C1 domain of Munc13-1 binds diacylglycerol (DAG), which helps membrane localization of this protein and promotes vesicle fusion, facilitating synaptic vesicle release. Previously, it was shown that ethanol binds to the C1 domain of Munc13-1 in vitro at concentrations below 100 mM (Das et al., 2013). The ethanol binding inhibits DAG binding to the Munc13-1 C1 domain at a concentration as low as 25 mM (Xu et al., 2017). Previously, it was also found that *Dunc13*, which is the Drosophila homolog of the mammalian *Munc13-1*, haploinsufficiency showed high-level resistance to the sedative effect of ethanol. This result was initially unexpected since overall Dunc13 activity is lower in the *Dunc13* haploinsufficient flies. We predicted this would sensitize the flies to further Dunc13 inhibition by ethanol, leading to more rapid sedation. One possible mechanism is that reducing *Dunc13* activity genetically, through the expression of *Dunc13* RNAi transgenes or mutation, will mimic the molecular changes that accompany ethanol tolerance. Here we showed that flies with chronically reduced *Dunc13* activity produced significantly more rapid tolerance to the sedative effects of ethanol than wild type control flies. In addition, we analyzed the genes which were differentially expressed after ethanol treatment. Here we showed the genes which might be responsible for

inducing rapid tolerance and the patterns of transcriptional changes were largely different between *Dunc13* haploinsufficiency group and ethanol-treated group.

DEDICATION

This thesis is dedicated to everyone who supported my decision to study biology. In particular, I thank my family and my friends for encouraging me during my own times of stress and anxiety.

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CHAPTER I.

INTRODUCTION

The exposure to intoxicating levels of ethanol results in tolerance to the sedative effects of this drug (Berger, Heberlein, & Moore, 2004; Henrike Scholz, Jennifer Ramond, Carol M. Singh, & Ulrike Heberlein, 2000). This tolerance is likely a necessary step in the formation of alcohol dependence (H. C. Becker, 2008). The formation of tolerance is predicted to occur through a homeostatic response to the neural inhibition induced by ethanol, but the exact mechanisms by which this occur remain largely unknown (Most, Ferguson, & Harris, 2014). The overarching goal of my project has been to better understand the role of the Dunc13 active zone protein in ethanol tolerance formation.

1. The importance of understanding the mechanism of tolerance formation

When alcohol tolerance develops, animals are more likely to consume a larger amount of alcohol so that they can acquire the same level of hedonic effect of alcohol as previously (Bell, 1994). The consumption of higher amounts of alcohol and repeated consumption of alcohol has been proposed to create an increased level of neuroadaptation that opposes the effect of the drug, which represents the state of tolerance (A. Ghezzi & Atkinson, 2011). After the drug clears the system, the neuroadaptation for the drug still remains, resulting in a withdrawal state (A. Ghezzi & Atkinson, 2011). This withdrawal state is unbalanced, in which only the neuroadaptation exists and the nervous system is susceptibility to seizures, and individuals may suffer from high

levels of psychological distress (Littleton, 1998). During a withdrawal state, animals are more likely to consume more alcohol, which can rebalance the neuroadaptation and remove the withdrawal symptoms (Littleton, 1998).

Alcohol Use Disorder (AUD) is caused by compulsive drinking. People are diagnosed as AUD when they meet the criteria on the Diagnostic and Statistical Manual of Mental disorders (DSM-5), asking how much they are dependent on alcohol. Based on the statistics from the 2015 National Survey on Drug Use and Health (NSDUH), 4.7 percent of people in the U.S. had AUD. AUD leads people to drink excessive amounts of alcohol, resulting in both mental and physical problems. Ultimately, this disease causes many kinds of loss in our society. Specifically, Sacks, J. J., et al. estimated the total cost that is caused by excess drinking on categories such as health care, lost productivity, and crime to be $249.0 billion (Sacks, Gonzales, Bouchery, Tomedi, & Brewer, 2015).

Although the negative impacts of excessive alcohol intake are a significant problem in the U.S., many of the molecular mechanisms involved in the development of alcohol tolerance remain largely unknown. A better understanding of the role of *Dunc13* in ethanol tolerance formation will provide new knowledge about ethanol tolerance formation, which can be applied to the prevention of alcohol dependence.

2. Why is *Drosophila* used for alcohol use disorder research?

Drosophila has been an excellent model organism for the genetic dissection of complex biological processes for over a century for research in biology (Jeibmann & Paulus, 2009; Stephenson & Metcalfe, 2013). Their short life cycle from egg to adult is approximately 12 days to complete, and its lifespan is about 70 days at room temperature (Linford, Bilgir, Ro, &

Pletcher, 2013). In the lifetime of female flies, they lay from 750 to 1,500 eggs. A short life cycle and a large number of offspring are advantageous for genetic research since those features allow us to create new fly lines and expands the designed fly line in a short period of time.

The genome of *Drosophila* is simple and more easily manipulable, also making it a strong genetic model system. The length of the *Drosophila* genome is relatively short. The length is 180 million base pairs, and the genome has 13,601 genes, with reduced redundancy compared to more complex vertebrates (Adams et al., 2000). The length of the human genome is 3 billion base pairs, and the genome has 25,000 genes, much more complex than *Drosophila*. In addition, of the 2,309 human disease-genes, approximately 700 genes are estimated to be well-conserved homologs in *Drosophila (Bier, 2005)*.

There are several techniques that allow us to manipulate the genome to investigate gene functions. In this study, two different techniques were used. First, the *Dunc13*^{P84200} mutation is caused by the insertion of transposable P-element (Aravamudan, Fergestad, Davis, Rodesch, & Broadie, 1999). As a result, the level of expression of the targeted gene is decreased (Aravamudan et al., 1999). Second, the *Gal4*-UAS system was used to decrease the expression of *Dunc13*. UAS-*Gal4* system is for activating gene expression in a specific tissue (Brand & Perrimon, 1993; Duffy, 2002). Upstream activation sequence (UAS). *Gal4* specifically binds to UAS and activates the UAS, and the gene of interest t is expressed in cells defined by the Gal4 expression. In this study, UAS-RNAi transgenes for *Dunc13* were used to decrease the expression of Dunc13.

Drosophila has been used as an effective model for health-related studies, including AUD studies since *Drosophila* and humans share approximately 75% homology in disease-causing genes (Malherbe, Kamping, Delden, & Zande, 2005) and, since it is relatively easy to

validate the behavioral roles of the genes, *Drosophila* is used as model for the genomic studies of AUD (Gregory L. Engel, Kreager Taber, Elizabeth Vinton, & Amanda J. Crocker, 2019). For example, *Drosophila* possesses *Alcohol dehydrogenase (Adh)* gene that expresses Adh to metabolizes alcohol to catalyzes the oxidation of alcohols to the aldehyde in their body, resulting in alcohol resistance (Gregory L. Engel et al., 2019; Malherbe et al., 2005; Winberg & McKinley-McKee, 1998). Many alcohol-related genes have been either first described or validated in *Drosophila* (Gregory L Engel, Kreager Taber, Elizabeth Vinton, & Amanda J Crocker, 2019; Petruccelli & Kaun, 2019).

3. Ethanol resistance and tolerance

Ethanol resistance occurs in an organism that is naive to the effect of ethanol and indicates that an organism requires a larger amount of ethanol for the same level of response to the drug compared to the other organisms (Atkinson, 2009). There are two ways to develop resistance to drugs. One way is to reduce the sensitivity to the effect of drugs by altering the sensitivity of the drug's binding site or to create another pathway to compensate for the pathway that is disrupted by the drugs (Fry, 2014). The other way is to prevent the drugs from reaching the target organs by detoxification, excretion, sequestration, or reducing absorption (Fry, 2014).

Ethanol tolerance, on the other hand, is a resistance induced by prior drug exposure. In other words, decreased sensitivity to the sedative effects of ethanol after first exposure of ethanol (Atkinson, 2009; A. Ghezzi & Atkinson, 2011). Tolerance has been categorized into metabolic (pharmacokinetic) tolerance and functional (pharmacodynamic) tolerance based on the mechanisms to develop tolerance. Metabolic tolerance is achieved through the more efficient

removal of alcohol from the body, and functional tolerance relies on changes in neural function (Atkinson, 2009; Berger et al., 2004; Fadda & Rossetti, 1998).

There are three types of tolerance in *Drosophila*: acute, rapid, and chronic (H. Scholz, J. Ramond, C. M. Singh, & U. Heberlein, 2000). Acute tolerance is developed during drug exposure. Rapid tolerance is induced after single drug exposure and chronic tolerance arises after multiple or long-term continuous exposures (Berger et al., 2004; Kalant, LeBlanc, & Gibbins, 1971). In this study, we focused mainly on rapid tolerance.

4. Rapid tolerance

Rapid tolerance is induced by single and short-term intoxicating ethanol exposure, and after the ethanol has been metabolized. In *Drosophila*, intoxicating doses of ethanol can be achieved by placing the flies within a stream of ethanol vapor. In the study from Berger, Heberlein, & Moore, 2004, rapid tolerance was induced 6 hours after the 30 minutes exposure of 60% ethanol vapor. The rapid tolerance was maximum at 6 hours after the ethanol exposure and remain significant until 24 hours after the first exposure (Berger et al., 2004). In the study from Scholz, Ramond, Singh, & Heberlein, 2000, rapid tolerance was induced by exposure to 53% ethanol vapor (Ethanol/Air = 50/ 45) until they were intoxicated (eluted from an inebriomter apparatus), and then tolerance was measured after 4 hours. Although they observed that the tolerance was maximum after 2 hours of incubation, 4 hours of incubation were used for behavioral experiments because tolerance was measurable and the flies had enough time to eliminate all absorbed ethanol and rehydrate and feed (Henrike Scholz et al., 2000).

5. Neural requirements for ethanol sensitivity and rapid tolerance

Several *Drosophila* brain regions have been important in ethanol sensitivity and the development of tolerance, including the ellipsoid body. The expression of tetanus toxin light chain, which inhibits synaptic vesicle release by cleaving synaptobrevin, in the ellipsoid body inhibited the formation of rapid tolerance (Henrike Scholz et al., 2000). Moreover, it was found that the homer scaffold protein was required within the ellipsoid body for the formation of ethanol sedation tolerance, indicating these neurons play an essential role in this process (Nancy L. Urizar, 2007).

Rapid tolerance formation also requires octopamine. The Tbh_{8} mutants, which are defective in Tyramine β-hydroxylase and are severely depleted in the synthesis of octopamine, failed to develop rapid tolerance (Berger et al., 2004; Henrike Scholz et al., 2000). Octopamine is the invertebrate equivalent to noradrenaline (Bauknecht & Jékely, 2017; Monastirioti, Linn, & White, 1996; Wallace, 1976). Interestingly, it was also shown that blocking the activity of the noradrenergic system in mice inhibited functional ethanol tolerance, suggesting a conserved role for these monoamines (Ritzmann & Tabakoff, 1976; Tabakoff & Ritzmann, 1977). Based on these studies, a normal level of the release of neurotransmitter, octopamine synthesis, and noradrenergic system activation, in the brain are necessary to develop rapid tolerance.

6. Chronic tolerance

Chronic tolerance is induced after multiple or long-term continuous exposures of ethanol. The concentration of ethanol should be lower than that of rapid tolerance. The duration of chronic tolerance is longer than rapid tolerance. Importantly, chronic tolerance requires new protein synthesis (Berger et al., 2004). It was shown that when the flies were treated with

cycloheximide, which inhibits protein synthesis by blocking the translocation process, chronic tolerance was inhibited, but rapid tolerance was not inhibited (Berger et al., 2004).
Chronic tolerance was induced by 48 hours of 11% ethanol exposure (Berger et al., 2004). Chronic tolerance lasts 48 hours after the first exposure, while rapid tolerance lasts only 24 hours after the first exposure. In chronic tolerance, pharmacokinetic alteration is not involved because long-term exposures of ethanol vapor did not change the level of ethanol concentration in the *Drosophila* body(Berger et al., 2004). The rate of ethanol metabolism and clearance did not change with the formation of chronic tolerance (Berger et al., 2004).

7. The synaptic release regulator Munc13 has a role in ethanol tolerance

Chemical neurotransmitters are stored in synaptic vesicles in the presynaptic neuron (Hilfiker et al., 1999). Many proteins are assembled at the active zone that regulates the fusion of the synaptic vesicles with the presynaptic membrane (Kavalali, 2015). The core proteins that associate with the synaptic vesicle are N-ethylmaleimide sensitive factor (NSF), soluble NSF attachment proteins (SNAP), SNAP receptor (SNARE) proteins, Munc18, Munc13, and Rab3 (Südhof & Rizo, 2011; Y. Wang & Okamoto, 1997). There are mainly three types of SNARE proteins, which are the vesicle-associated membrane proteins (VAMP; also known as synaptobrevin), syntaxin, and synaptosome associated protein that is also known as SNAP25 (Lang, Margittai, Hölzler, & Jahn, 2002).

Munc13-1, the mammalian ortholog to Dunc13, is a presynaptic active-zone protein that is essential for synaptic vesicle fusion (Betz et al., 1998; Betz, Okamoto, Benseler, & Brose, 1997; Rhee et al., 2002). Munc13-1 interacts with the vesicle fusion machinery at active zones in the mammalian brain (Rizo & Xu, 2015). The C1 domain of Munc13-1 binds diacylglycerol

(DAG), which helps the membrane localization of this protein facilitating synaptic vesicle release (Augustin, Rosenmund, Sudhof, & Brose, 1999; Varoqueaux et al., 2002). Previously, it was shown that ethanol binds to the E_{582} residue in the C1 domain of Munc13-1 *in vitro* (Das et al., 2013). DAG, binds to His_{567} in the C1 domain of Munc13-1 (Basu, Betz, Brose, & Rosenmund, 2007). The distance between the ethanol binding site of E_{582} residue and the diacylglycerol (DAG) binding site of His_{567} is only 8.8A (Das et al., 2013). Ethanol binding to the C1 domain of Munc13-1 inhibits DAG binding, which is predicted to reduce Munc13-1 activity (Xu et al., 2018). We hypothesize that the inhibition of Munc13-1 activity by ethanol, which should reduce presynaptic activity, will have functional consequences in the development of tolerance.

To test the role of ethanol-Munc13-1 interaction on ethanol's impact on the nervous system, the Roman lab has been using *Drosophila* as a model system. The sensitivity to ethanol in flies can be measured using the inebriometer and the FlyBar, an apparatus that quantifies the loss of postural control induced by ethanol exposure (Heberlein, Wolf, Rothenfluh, & Guarnieri, 2004; van der Linde, Fumagalli, Roman, & Lyons, 2014). The *Dunc13* gene is the *Drosophila* ortholog to Munc13-1and has a conserved C1 domain (Aravamudan, Fergestad, Davis, Rodesch, & Broadie, 1999). Flies haploinsufficient for *Dunc13* (heterozygous for the $Dunc13^{P84200}$ loss-of-function allele) were found to be behaviorally resistant to sedating concentrations of ethanol (Xu et al., 2018). This resistance to ethanol sedation phenotype was rescued by expressing the rat Munc13-1 within the fly brain, demonstrating functional complementation between Dunc13 and Munc13-1 and demonstrating the phenotype is due to a reduction in *Dunc13* activity (Aravamudan et al., 1999; Das et al., 2013; Shiyu Xu et al., 2018).

In *Drosophila*, the injection of sedating levels of ethanol leads to a significant and robust reduction in synaptic vesicle fusion compared to the vehicle-injected flies; however, presynaptic membrane depolarization and Ca^{2+} influx were not affected by ethanol (Xu et al., 2018). From these results, it can be inferred that the ethanol impacts active zone processes independent of early presynaptic activation events, consistent with a role of Dunc13 in mediating these physiological effects of ethanol. Moreover, it was found that flies haploinsufficient for *Dunc13*, with reduced *Dunc13* expression, displayed resistance to the ethanol-induced inhibition of synaptic vesicle fusion (Shiyu Xu et al., 2018). Furthermore, reducing the expression of *Dunc13* by expressing RNAi dramatically reduced the ethanol sedation sensitivity (Shiyu Xu et al., 2018). Thus, reducing *Dunc13* activity leads to a behavioral resistance to the sedative effects of ethanol. Hence, chronically reducing *Dunc13* activity results in synaptic resistance, as well as behavioral resistance, to the effects of sedating concentrations of ethanol. The reduced sensitivity of *Dunc13* haploinsufficient flies is counter to the naive prediction that reducing *Dunc13* activity genetically would lead to increased neural inhibition and sedation by sensitizing the flies to the effects of ethanol on *Dunc13* activity. However, the results are consistent with the hypothesis that reduced *Dunc13* activity results in a synaptic homeostasis response that leads to ethanol tolerance.

When flies are exposed to high levels of ethanol, they form functional tolerance (Henrike Scholz et al., 2000). The homeostasis response involves functional tolerance (G. W. Davis & Muller, 2015). Functional tolerance is defined as a decrease in drug responsiveness formed by prior drug exposure that relies on changes in the neuronal activity (Henrike Scholz et al., 2000). A single pre-exposure to ethanol results in greater resistance to a second ethanol exposure performed a few hours later (Henrike Scholz, Jennifer Ramond, Carol M. Singh, & Ulrike

Heberlein, 2000). Functional tolerance has been proposed to originate in homeostatic changes in neural transmission (Koob & Bloom, 1988; Park, Ghezzi, Wijesekera, & Atkinson, 2017). There are several important mechanisms beyond *Munc13* inhibition that explain, in part, the homeostatic change. It has been shown that CaV2.1, which is a voltage-dependent Ca^{2+} channel, adjusts the Ca^{2+} concentration in the presynaptic region when the glutamate receptor is inhibited (Frank, Kennedy, Goold, Marek, & Davis, 2006; Müller & Davis, 2012). Deficits such as the decreased activity of glutamate receptor increased release-ready vesicles and the amount of active zone protein such as Bruchpilot that facilitate efficient vesicle release also may account for homeostatic responses to high concentrations of ethanol (Müller, Liu, Sigrist, & Davis, 2012).

8. Other genes contributing to ethanol tolerance

In *Drosophila*, the formation of chronic ethanol tolerance requires transcriptional changes in several presynaptic proteins, including Synapsins, Dynamin, Homer, Integrins, BK channels, Syntaxin 1A, and the GABA B receptor. (Cowmeadow, Krishnan, & Atkinson, 2005; Ghezzi & Atkinson, 2011). Moreover, it was shown that exposure to ethanol vapor leads to changes in the histone acetylation of these genes (Ghezzi et al., 2013).

9. The role of BK channels in ethanol tolerance

The main function of the BK channels is to allow the flow of large amounts of K^{+} across the cell membranes out of the cell after depolarization (Lee & Cui, 2010; Yuan, Leonetti, Pico, Hsiung, & MacKinnon, 2010). The BK channel speeds up the repolarization after depolarization of action potentials (A. Ghezzi & Atkinson, 2011; Alfredo Ghezzi, Pohl, Wang, & Atkinson,

2010). Therefore, BK channels in the presynaptic region make refractory period short and enhance the capacity for repetitive firing (A. Ghezzi & Atkinson, 2011). This role of BK channels to increase the firing frequency is important in alcohol tolerance formation since alcohol exposure decreases the firing frequency(A. Ghezzi & Atkinson, 2011).

The *slowpoke* gene (*slo*) encodes the *Drosophila* BK Channel (Yazejian et al., 1997). The expression of *slowpoke* increases after the exposure to alcohol, which results in an increase in firing frequency and compensates for the reduction in firing frequency created by ethanol (Roshani B. Cowmeadow et al., 2006; Alfredo Ghezzi et al., 2010). A genomic survey of histone H4 acetylation (H4Ac) using the chromatin-immunoprecipitation assay (ChIP-chip) confirmed that histone acetylation of *slowpoke*, which is a gene implicated in the production of ethanol tolerance, was upregulated after exposure to ethanol. Loss-of-function mutations significantly blocked the formation of tolerance to ethanol, demonstrating a requirement for *slowpoke* in this process (Ghezzi et al., 2013).

In mammals, the BK channel is expressed throughout central and peripheral tissues. BK channel expression is high in aorta and brain but low in heart skeletal muscle, kidney, spleen, and lung. Specifically, the hippocampus and the corpus callosum have a high amount of BK channel (Tseng-Crank et al., 1996). The activity of the mammalian slo channel has also been found to be sensitive to ethanol (Brodie, Scholz, Weiger, & Dopico, 2007; Dopico, 2003). Moreover, the expression of the BK β4 subunit in mice controls behavioral tolerance to the sedative effects of ethanol (Gilles E. Martin et al., 2008). The BK channel is composed of α subunits only or α subunits and four different β subunits. The α subunit, which is expressed by *KCNMA1*, is the pore-forming unit. The β subunits, which are expressed by *KCNMB1-4*, have a regulatory function, and the activity of the BK channel depends on the association between α and

β subunits. For example, BK channels that contain the β1 subunit have higher sensitivity to Ca^{2+} by changing the conformation of the binding site for Ca^{2+} (Cox & Aldrich, 2000). The β2 and β3 subunits have a role in reducing the BK channel activity by decreasing the current. It has been shown that when either β2 or β3 subunit was co-expressed with the α subunit, current was eliminated significantly faster than without the β2 and β3 subunits (Wallner, Meera, & Toro, 1999; Xia, Ding, & Lingle, 1999). The β4 subunit decreases the activity of the BK channel by slowing the time constant for activation of the BK channel; hence, it takes longer to activate BK channels with the co-expression of β4 subunits. Moreover, BK channels with β4 subunits require a higher level of depolarizing voltage to open the BK channel (Weiger et al., 2000).

Martin, G. E., et al., 2008 investigated the effects of ethanol on the BK channel by using HEK cells and mice (Gilles E. Martin et al., 2008). The β4 subunit was a focus of these studies since it had been previously shown that ethanol affected the somatic BK channel by increasing open probability (Martin et al., 2004). It was found that the open probability of BK channels consisting of αβ4 subunits significantly increased under ethanol exposure, and activity increased by 2.5 fold (Gilles E. Martin et al., 2008). Although ethanol exposure increased the open probability of BK channel consisting of only α subunits, the activity of the potentiated BK channel decreased after 5 minutes of ethanol exposure (Gilles E Martin et al., 2008). In mice, they investigated the physiological and behavior induction of rapid tolerance using β4 knock out (β4 KO) mutant mice. Interestingly, they found that under conditions where β4 is expressed, little tolerance of spiking activity was found in striatum slices or isolated medium spiny neurons, however, rapid tolerance was readily observed in the β4 KO mice, suggesting that β4 expression limits tolerance (Gilles E Martin et al., 2008). Furthermore, the β4 KO mice display stronger levels of rapid tolerance in behavioral locomotor assays (Gilles E Martin et al., 2008). Since the

β4 subunit limits the activity of BK channels, and the genetic loss of this subunit increases rapid tolerance formation, these data are consistent with a model of increases in BK activity being responsible for functional ethanol tolerance in mice.

In *Drosophila*, the *slowpoke* (*slo*) BK channel exists in the cortex of the brain, including neuronal cell bodies and neurites (M. N. Becker, Brenner, & Atkinson, 1995). The expression is specifically high in the mushroom bodies, centers for olfactory learning and sensory integration, and in the optic lobes (M. N. Becker et al., 1995). The *slo* channel is found in flight muscle that is used for controlling their wings for their flight (M. N. Becker et al., 1995).

It has been shown that in *Drosophila*, the BK channel plays an important role in inducing ethanol tolerance since loss-of-function mutations in *slo* fail to form ethanol sedation tolerance (R. B. Cowmeadow, Krishnan, & Atkinson, 2005). The *slo4* mutation is a chromosomal inversion that disrupts the *slowpoke* gene (Atkinson, Robertson, & Ganetzky, 1991; M. N. Becker et al., 1995). The *ash218* and *87-5* mutations carry a large deletion on the 3rd chromosome that includes a portion of the *slowpoke* promoter region (Atkinson et al., 2000). Although *slo4* heterozygous acquired the tolerance, *slo4* homozygous mutant and *slo4* transheterozygotes over the *87-5* and a*sh218* deletions did not acquire tolerance (R. B. Cowmeadow et al., 2005; G. W. Davis & Muller, 2015; A. Ghezzi & Atkinson, 2011). These results indicate that the *slo* BK channel is required in the formation of ethanol tolerance in *Drosophila*. Subsequently, it was shown that increasing *slowpoke* expression induces resistance to the sedative effects of ethanol (Roshani B. Cowmeadow et al., 2006). In this experiment, an HSP70 heat-shock promoter was used to control the expression of *slo*. Heat-induced *slo* expression resulted in flies behaviorally resistant to ethanol sedation (Roshani B. Cowmeadow et

al., 2006). Hence, in *Drosophila*, the BK channel expressed by the *slowpoke* gene is necessary and sufficient for ethanol tolerance.

In *Caenorhabditis elegans*, SLO-1, which is the BK channel ortholog, exists in neuromuscular junctions in body wall muscle and pharyngeal muscle (Z.-W. Wang, Saifee, Nonet, & Salkoff, 2001). The structure of the nervous system of *C. elegans* is composed of neurons in the pharynx, head ganglia, where its brain exists, dorsal cord, ventral cord, and tail ganglia. SLO-1 exists in neurons, but especially SLO-1 is highly expressed in the nerve ring, where sensory axons make synaptic connections with interneurons (Z.-W. Wang et al., 2001). When *C.elegans* is exposed to ethanol, they move slower, bend less, and lay fewer eggs compared to normal condition (Davies et al., 2003). The behavioral sensitivity of *C. elegans* to ethanol was significantly increased in *slo-1* loss of function mutation compared to wild type (Davies et al., 2003; S. J. Davis, Scott, Hu, & Pierce-Shimomura, 2014). Furthermore, when *slo-1* was expressed in the neuron in *slo-1* loss of function mutant by using a tissue-specific gene promoter, the sensitivity to ethanol returned to the normal level (Davies et al., 2003). Therefore, they confirmed that *slo-1* expression was specifically required in neurons for normal ethanol sedation sensitivity (Davies et al., 2003). Moreover, they showed that ethanol increases the activity of SLO-1 in sensory and motor neurons by measuring the current when *C.elegans* was exposed to ethanol (Contet, Goulding, Kuljis, & Barth, 2016).

10. The gene expression of *sir2* in ethanol tolerance formation

In other studies, short-term exposure to ethanol was found to significantly decrease the expression of RNA for the *Drosophila* Sirt1 homolog *sir2*, affecting both the locomotor and postural control response to ethanol (Kong et al., 2010; Morozova, Anholt, & Mackay, 2006). In

addition, it was also shown that when *Drosophila* is exposed to ethanol vapor, the expression of *sir2,* which is required in mushroom bodies for the development of ethanol tolerance, ethanol preference, and ethanol reward, is decreased (Engel et al., 2016). On the other hand, the level of acetylation of *sir2* was increased to compensate for the decrease in gene expression of *sir2* (Engel et al., 2016). Importantly, although the expression of syntaxin, a presynaptic phosphoprotein that binds synaptic vesicles and regulates their dynamics, was decreased after exposure of ethanol, the expression of *syn* in *sir2* mutant flies was not decreased after exposure of ethanol (Engel et al., 2016). Thus, changes in the regulation of presynaptic proteins in response to ethanol may be responsible for altering synaptic physiology to compensate for ethanol's impact on presynaptic function.

I hypothesized that when ethanol binds to Dunc13, it results in the inhibition of presynaptic activity, which induces homeostatic changes in neurons that lead to chronic tolerance to the sedating properties of ethanol. I predicted that reducing *Dunc13* activity genetically, in heterozygotes for the loss-of-function $Dunc13^{P84200}$ allele or through the expression of *Dunc13* RNAi transgenes, will mimic the transcriptional changes in presynaptic proteins found during ethanol tolerance formation and that these same changes that are required for tolerance formation are also required for the ethanol resistance phenotype found after reducing *Dunc13* activity genetically. This prediction was to be addressed with the following aims:

Aim 1: Determine if a genetic reduction of *Dunc13* activity leads to a change in rapid tolerance formation.

Aim 2: Determine if a genetic reduction of *Dunc13* activity alters the transcriptional response to sedating levels of ethanol.

CHAPTER II.

METHODOLOGY

1. Fly stocks and Genetics

All flies were maintained on standard *Drosophila* food at 25°C on a 12/12 h light/dark cycle. Canton-S background (Cs) line was used as the wild type control in this experiment. *C819*-Gal4 (FBti0018454), *5.30*-Gal4 (FBti0148845), *Ruslan*-Gal4 (FBti0027486), *Dunc13*$_{JF02440}$ RNAi lines, and w^{+}; +; *ry*$_{506}$; *Dunc13*$_{P84200}$/*ci*$_{D}$ (FBst0300878) were provided by Bloomington Drosophila Stock Center. *Dunc13*$_{P84200}$/+ heterozygotes were generated by crossing virgin females of *ry*$_{506}$, with w^{+}; +; *ry*$_{506}$; *Dunc13*$_{P84200}$/*ci*$_{D}$ males. In all the experiments, 3-5 days old male flies were used.

2. Ethanol sedation

To measure ethanol resistance and ethanol tolerance, the Loss of Righting Reflex (LOR) assay was performed (Kim van der Linde, Emiliano Fumagalli, Gregg Roman, & Lisa C. Lyons, 2014). The ethanol vapor was generated by bubbling fresh air through two flasks, the first containing 100 % ethanol and the second tap water. The temperature of the two flasks was kept at 27℃. The airflow rate was 500 mL/min for each flask. Male flies were collected and kept in a food vial at 25℃ for 24 hours prior to behavioral testing. Before they were exposed to ethanol vapor, the flies were transferred to a new empty vial. They were then exposed to 50% ethanol vapor. The number of sedated flies were counted at 5 min intervals. The flies were counted as

being sedated when they fell onto their back or side for 5 seconds or beating their wings without flying. To induce rapid tolerance, the flies were exposed to the ethanol vapor until 90% of flies in the vial are sedated (1_{st} exposure). After the 1st exposure, the flies were transferred to vials containing food and kept at 25℃ for 4 hours. After 4 hours, they were transferred to an empty vial and then exposed to 50% ethanol vapor (2_{nd} exposure). The level of ethanol resistance of the naive group (naive group in figure 1) was determined by measuring the time to 50% LOR at 1_{st} exposure. To find the time to 50% LOR, the flies were exposed to the ethanol vapor until 50% of flies in the vial are sedated. The time to 50% LOR was calculated by using forecast function in Microsoft excel. The level of ethanol tolerance of the pre-exposure group (pre-exposure group in figure 1) was determined by measuring the time to 50% LOR at 2_{nd} exposure.

For the heat shock experiment, for all behavioral and molecular experiments, adult males that were 3-5 days old were used. The induction of gene expression was induced by placing the flies at 30℃ for 48 hours. This heat-shock allowed for Gal4 activity to be induced due to the loss of $Gal80_{ts}$. After the heat-shock, they were kept at 25℃ for 3 hours to allow the flies to recover. For the uninduced group, flies of each genotype were kept at 25℃ for 48 hours. They were kept for at 25℃ for 3 hours before the 1st exposure to ethanol. After the 1st exposure, the flies were transferred to vials containing food and kept at 25℃ for 4 hours. After 4 hours, they were transferred to an empty vial for Loss of Righting Reflex (LOR) assay.

3. RNA isolation

The 3 to 5 days old flies were exposed to 50% ethanol vapor by LOR assay. For the ethanol group, the flies were first exposed to 50% ethanol vapor for 25 minutes and transferred to vials containing food and kept at 25℃ for 4 hours. For the naive group, the flies were first

exposed to 100% water vapor for 25 minutes and transferred to vials containing food and kept at 25℃. In this procedure, there are four treatment groups: 1. no ethanol exposures mock treatments (Wild type, naive), and 2. Induction of ethanol tolerance (Wild type, ethanol). 3. no ethanol exposures mock treatments (*Dunc13*^{P84200}/+ heterozygotes, naive), and 4. Induction of ethanol tolerance (*Dunc13*^{P84200}/+ heterozygotes, ethanol). After 4 hours, they were frozen by liquid nitrogen, and their heads were separated. TRIzol RNA Isolation Reagents (ThermoFisher Scientific) were used to extract the RNA from heads of *Drosophila*.

4. RNA-seq

The isolated RNA samples were sent to Novogene (Sacramento, CA) for RNA-seq illumine data (San Diego, CA). (HiSeq platform, paired-end) The sequencing depth was x30. The size of the insert library was ~250bp.

5. Bioinformatics

The original raw data were transformed into sequenced reads. The raw data were recorded in a FASTQ file, which contains sequence information and corresponding sequencing quality information. The raw data were filtered by discarding reads with adaptor contamination, reads when uncertain nucleotides constitute more than 10 percent of either read (N > 10%), and reads when low-quality nucleotides (base quality less than 20) constitute more than 50 percent of the read. The HISAT2 alignment tool was used to map the filtered sequenced reads to the reference genome (Kim, Langmead, & Salzberg, 2015; Kim, Paggi, Park, Bennett, & Salzberg, 2019). The output from HISAT2 was obtained as SAM format files containing the individual reads alignment within the reference genome.

Differential gene expression was analyzed by using the read counts from gene expression level analysis. The read-counts of biological duplicates were normalized by DESeq (Anders & Huber, 2010). Negative binomial distribution was used to estimate the *p*-value in differential gene expression analysis. The adjusted *p*-value < 0.05 was used for differentially expressed gene screening standards. Gene ontology (GO) enrichment bar chart of differentially expressed genes (DEGs) was generated by GOSeq(Young, Wakefield, Smyth, & Oshlack, 2012) and topGO (Alexa & Rahnenfuhrer, 2007) (http://www.geneontology.org/). GO is an analysis to unify the presentation of gene and gene product involved in all species.

6. Statistical analysis

For the ethanol sedation data, the time to 50% LOR was measured once for each group and analyzed by two-way ANOVA (treatment x genotype) and unpaired *t*-test, followed by Tukey HSD post hoc tests.

CHAPTER III.

RESULTS

1. Reducing Dunc13 activity decreased ethanol sensitivity and increased tolerance

To investigate if genetically reduced *Dunc13* activity induces homeostatic changes that lead to chronic tolerance to the sedative effect of ethanol, we initially examined ethanol sensitivity and the rapid tolerance in flies heterozygous for the *Dunc13*^{P84200} loss-of-function mutation. *Dunc13* activity was reduced in *Dunc13*^{P84200}/+ heterozygotes (Das, et al. 2013). Sensitivity and rapid tolerance were determined as the amount of time required for 50% to reach the sedation criterium ($T_{1/2}$; (van der Linde et al., 2014)). Naive *Dunc13*^{P84200}/+ heterozygotes, never exposed to ethanol, were significantly less sensitive to the sedative effect of ethanol compared to wild type group in naive condition ($p < 0.05$) (Figure 1A, B). In addition, *Dunc13*^{P84200}/+ heterozygotes showed greater rapid tolerance to the sedative effects of ethanol at a second ethanol exposure ($p < 0.05$) (Figure 1A, B). Furthermore, the level of increase in rapid tolerance in the *Dunc13*^{P84200}/+ heterozygotes group was significantly higher than that of the wild type group (Figure 1C). This result suggested that a reduction in *Dunc13* activity by *Dunc13*^{P84200}/+ heterozygotes led to an increased rapid tolerance to ethanol sedation.

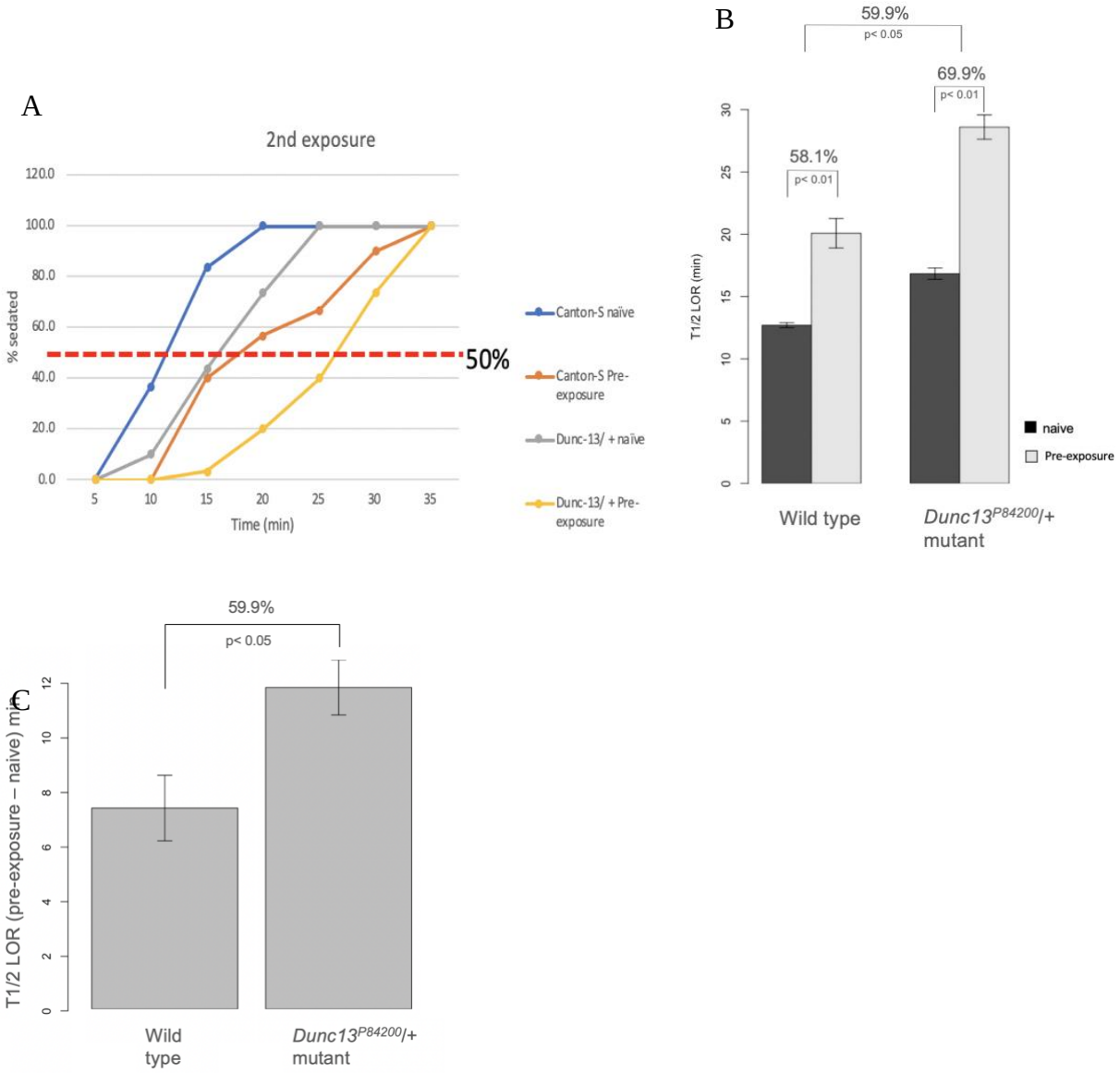


Figure 1. *Dunc13*$_{P84200}$/+ heterozygotes led to an increased rapid tolerance to ethanol sedation. (A) The number of sedated flies by ethanol vapor was measured every 5 minutes. There were shifts in % sedated flies between the naive group and the pre-exposure group in both wild type and *Dunc13*$_{P84200}$/+ heterozygotes groups. (B) Using the forecast function, the time at which 50% of the flies were sedated ($T_{1/2}$ LOR) was calculated, and the mean of $T_{1/2}$ LOR was calculated for each group. The $T_{1/2}$ LOR was increased by 58.1% in the pre-exposure groups of wild type, and the $T_{1/2}$ LOR was increased by 69.9% in pre-exposure groups of *Dunc13*$_{P84200}$/+ heterozygotes. *Dunc13*$_{P84200}$/+ heterozygotes showed greater ethanol tolerance compared to the wild-type flies (n = 12, $p < 0.05$). (C) The degree of induction of rapid tolerance after ethanol exposure was calculated by subtracting $T_{1/2}$ LOR of the naive group from $T_{1/2}$ LOR of the pre-exposure group and compared between the wild type and *Dunc13*$_{P84200}$/+ heterozygotes groups (t-test, n = 12, $p < 0.05$).

Next, *Dunc13* expression was decreased with *Dunc13*-targeted RNAi by using the GAL4-based TARGET gene expression system (Shiyu Xu et al., 2018). The TARGET system uses a transgene expressing a temperature-sensitive allele of Gal80 to inhibit the Gal4 transcription factor. Transcription is then activated through a heat shock, which inactivates the Gal80. We used *5.30*-Gal4, *Ruslan*-Gal4, and *C819*-Gal4 drivers to drive the expression of the UAS-*Dunc13* RNAi transgene. These three Gal4 drivers are specific to the ellipsoid bodies of the central complex substructure (Kong, Woo, et al., 2010; Krashes & Waddell, 2008). Since the ellipsoid body is an important brain structure for the development of rapid tolerance (Nancy L. Urizar, 2007), we selected these three ellipsoid body Gal4 lines to examine the role of *Dunc13* activity within these neurons.

Interestingly, we found that the heat-shock treatment we used to remove the Gal80 activity in the TARGET system, 30 °C for 48 hours, significantly decreased the ethanol sensitivity at 1st ethanol exposure and also rapid tolerance at 2nd ethanol exposure in wild type control group (Figure 2).

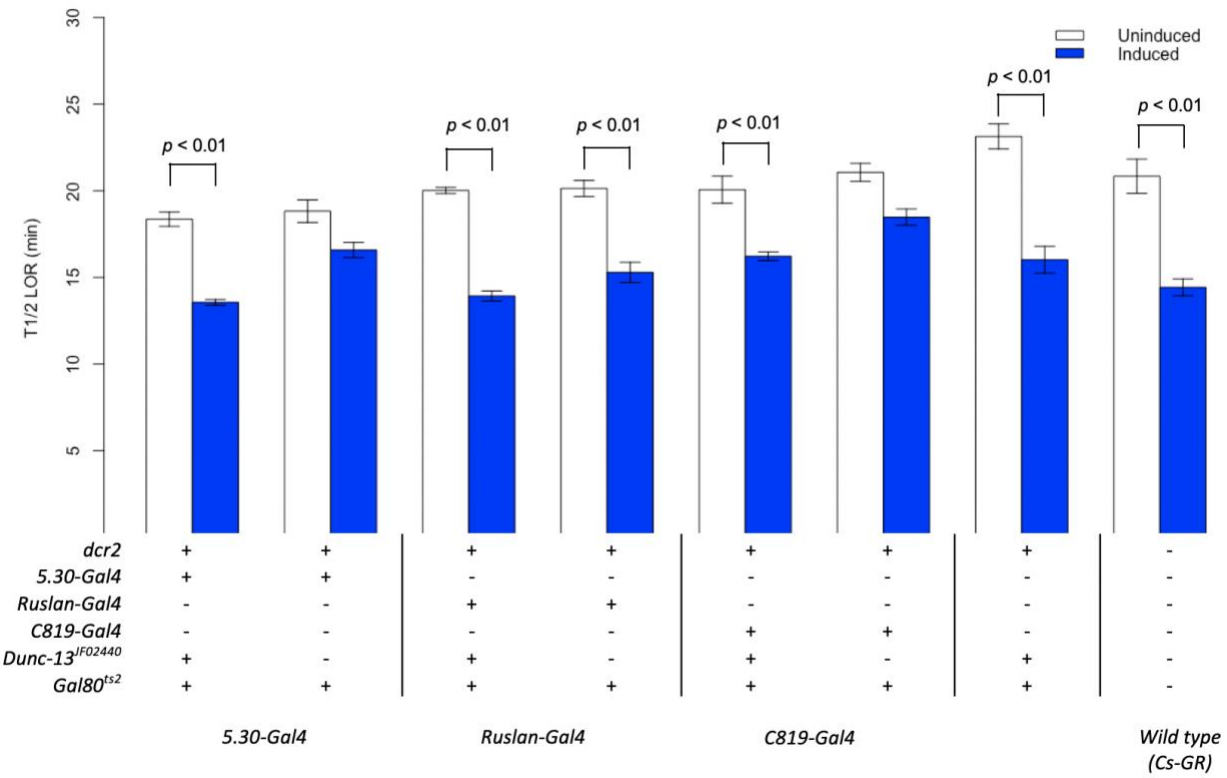


Figure 2. Heat shock led to a significantly faster $T_{1/2}$ **LOR.** Prior to the induction of rapid tolerance, the neural expression of the *Dunc13*^{JF02440} RNAi was induced by 30°C heat for 48 hours, followed by a 3 hours recovery period at room temperature. The $T_{1/2}$ LOR was measured after the rapid tolerance was induced. The protocol to induce rapid tolerance was in the methodology section. The heat-shock treatment led to a significantly faster $T_{1/2}$ LOR in experimental, genotype, and wild-type control ($p < 0.05$, $n = 12$).

Previous studies that used heat shock treatment did not report an effect of this temperature shift in ethanol sensitivity (Shiyu Xu et al., 2018) (Figure 3). The purpose of this experiment was to investigate the ethanol tolerance formation when *Dunc13* is reduced genetically within a targeted subset of the nervous system. However, the effect of the heat shock by itself resulted in a confounding effect. A possible explanation for the decrease in the ethanol resistance and ethanol tolerance was that a long period of heat shock treatment stressed the flies, which perhaps caused dehydration, and the flies could not recover enough before the behavioral

assays. Regardless of the mechanism, we cannot make a conclusion about the effect of *Dunc13* activity within the ellipsoid bodies from this experiment.

We need to generate a new protocol on heat shock experiment by changing the recovery time, heat time, or temperature of heat shock so that the heat shock *per se* does not cause a difference in the ethanol sensitivity and tolerance in wild type flies.

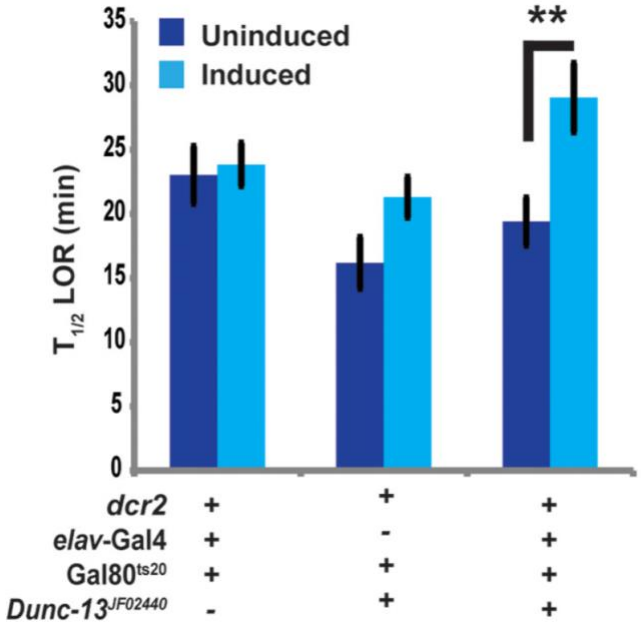


Figure 3. The effect of the heat shock did not result in a confounding effect on the ethanol resistance in the study from Shiyu Xu et al., 2018. 30°C heat treatment was added to the flies for 24 hours, followed by 3 hours of recovery period at room temperature. The control group without *Dunc-13*$_{JF02440}$ RNAi transgenes (group on the left) did not show significant change on the T1/2 LOR after the heat shock treatment ($p < 0.01$, n = 8) (Shiyu Xu et al., 2018).

2. Differential expression analysis

To determine if a genetic reduction of *Dunc13* activity alters the transcriptional response to sedating levels of ethanol and to identify possible genes that are responsible for the development of ethanol tolerance, we investigated gene expression levels in wild type and *Dunc13*^{P84200}/+ heterozygotes 4 hours after the exposure to either 50% ethanol vapor until 90% sedation or humidified air as a control. Differential gene expression in these experiments was initially analyzed using DEseq (Anders & Huber, 2010). Genes that had a significant differential expression (adjusted $p < 0.05$, 1,410 genes) were then clustered into groups based on their patterns of expression (Figure 4). Clustering genes with similar expression patterns helps us to investigate the genes of function. Genes in the same cluster are likely regulated in a similar manner and hence might have similar functions or share a biological process.

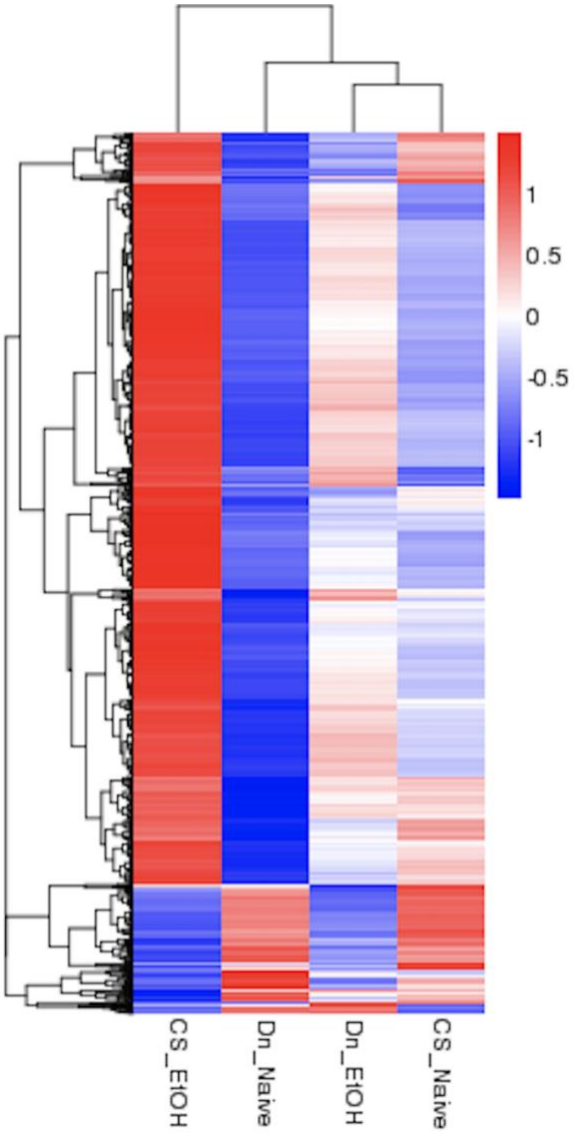
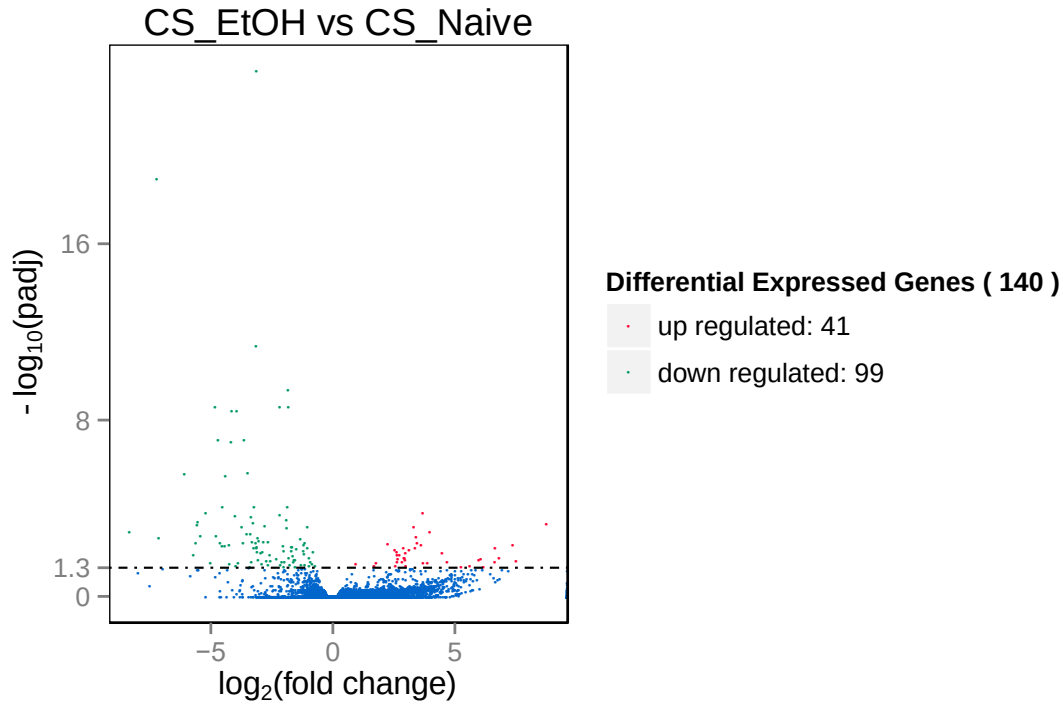


Figure 4. Cluster analysis of gene expression differences. Cluster analysis shows the genes with similar expression patterns under different experimental conditions. The genes differentially expressed were clustered using the $\log_{10}$(FPKM+1) value by hierarchical clustering. The dendrogram (top) shows the similarity of gene expression patterns among the groups. More related groups are arranged closely in the dendrogram. Red indicates genes with high expression levels. Blue indicates genes with low expression levels. CS_Naive: Wild type flies were exposed to 100% H_2O vapor for the same time as CS_EtOH group were exposed to ethanol vapor, and then kept in vials containing regular food for four hours at 25℃. CS_EtOH: Wild type flies were exposed to 50% ethanol vapor until 90% sedation, and then kept in vials containing regular food for four hours at 25℃. Dn_Naive: *Dunc13*^{P84200}/+ heterozygotes were exposed to 100% H_2O vapor for the same time as the Dn_EtOH group was exposed to ethanol vapor, and then kept in vials containing regular food for four hours at 25℃. Dn_EtOH: *Dunc13*^{P84200}/+ heterozygotes flies were exposed to 50% ethanol vapor until 90% sedation, and then kept in vials containing regular food for four hours at 25℃.

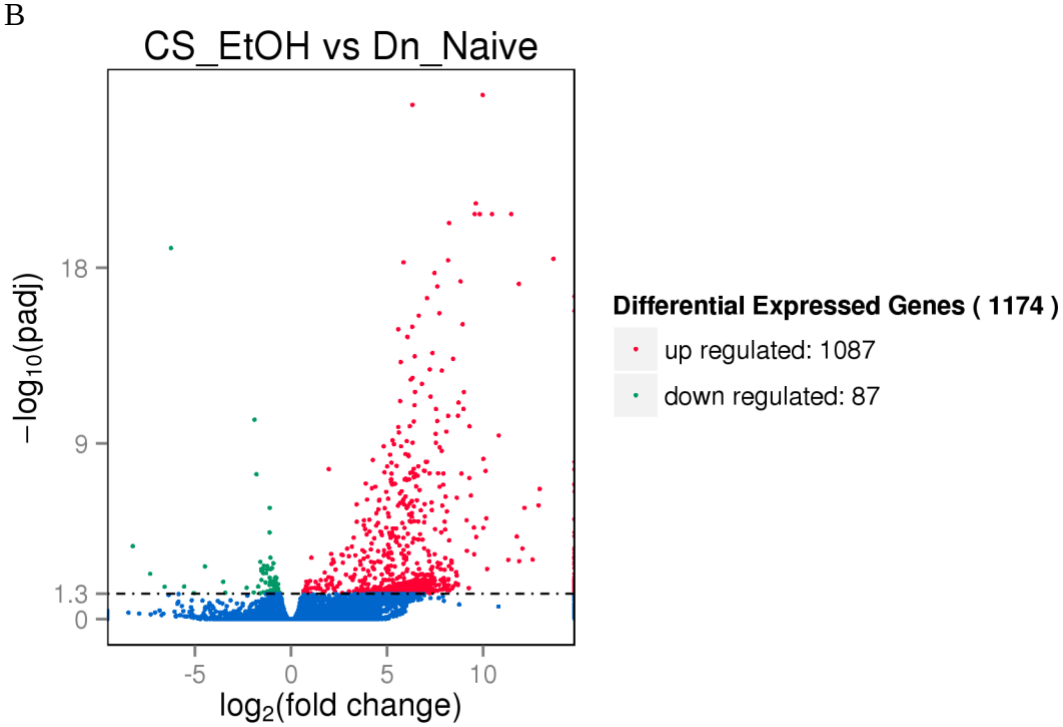
As an overall trend, *Dunc13*^{P84200}/+ heterozygotes had lower gene expression than wild type in both conditions of naive and ethanol. In comparing between naive *Dunc13*^{P84200}/+ heterozygotes and ethanol-treated wild type flies, large clusters of gene expression in those groups had distinctly different levels of gene expression. In other words, the high level of gene expression observed in naive *Dunc13*^{P84200}/+ heterozygotes were low in ethanol-treated wild type and vice versa. Interestingly, ethanol exposure of the *Dunc13*^{P84200}/+ heterozygotes brought the overall level of gene expression much closer than that of naive wild type flies, suggesting that ethanol help stabilizes the transcriptional activity of these *Dunc13*^{P84200}/+ heterozygotes.

To identify possible genes that are responsible for the development of ethanol tolerance, the role of genes that were up-regulated or down-regulated after ethanol exposure in Canton-S wild type flies were investigated (Figure 5; Table 1). In the discussion section, those genes which were differentially expressed are discussed in detail.

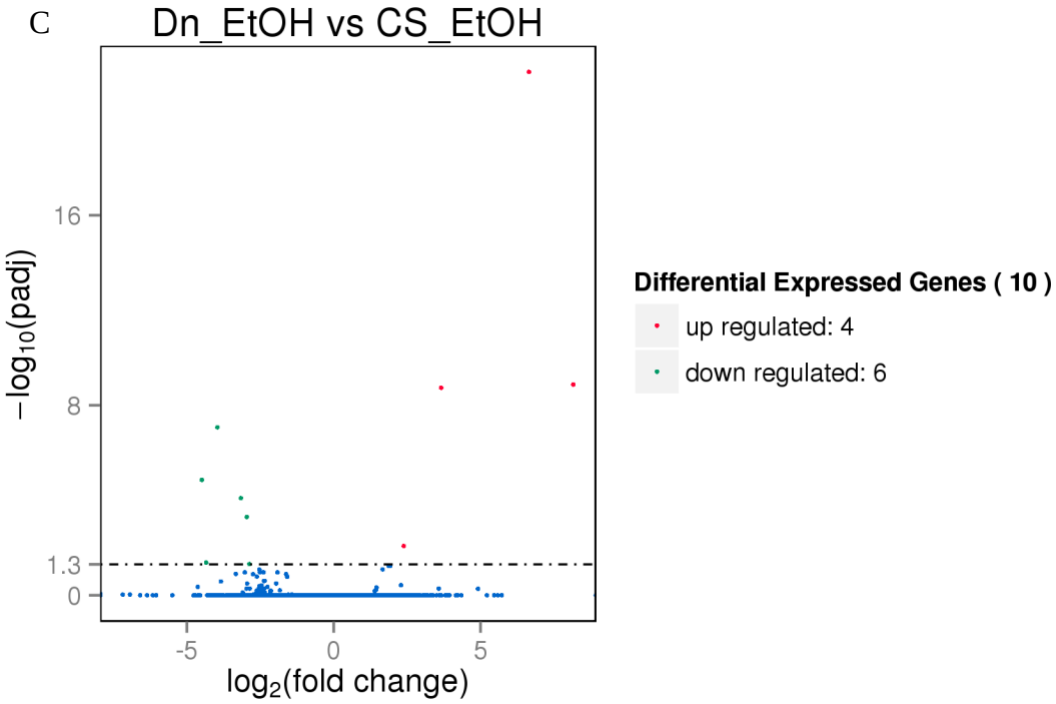
A


CS_EtOH vs CS_Naive
- log10(padj)
16
8
1.3
0
−5
0
5
log2(fold change)
Differential Expressed Genes (140)
up regulated: 41
down regulated: 99

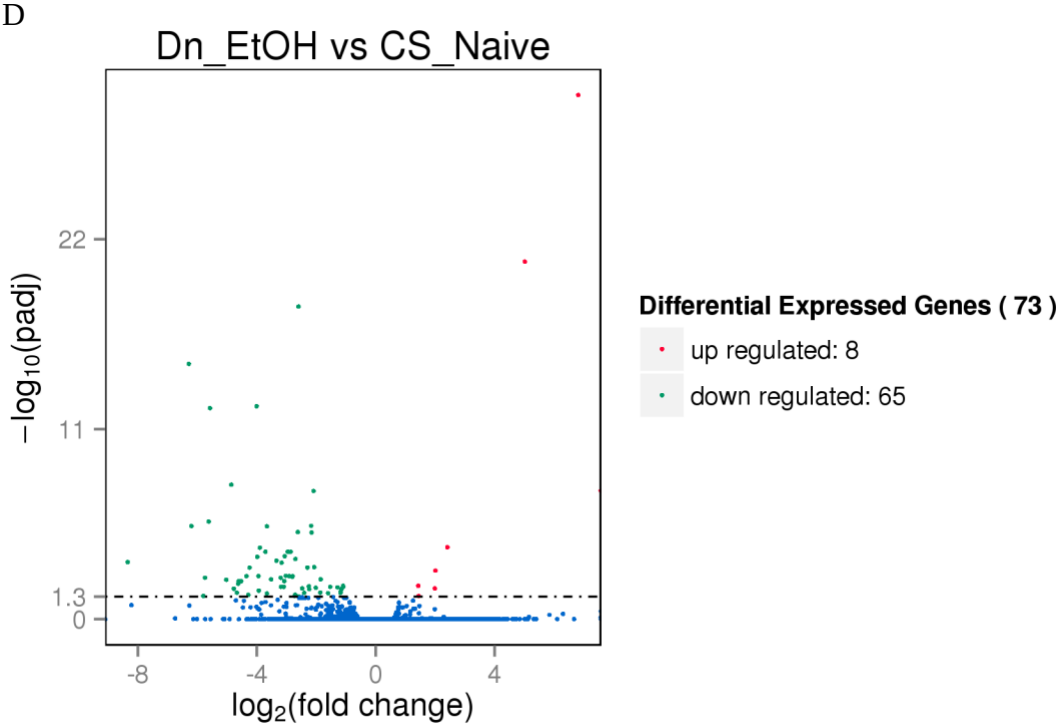
B


CS_EtOH vs Dn_Naive
−log10(padj)
18
9
1.3
0
-5
0
5
10
log2(fold change)
Differential Expressed Genes (1174)
up regulated: 1087
down regulated: 87

C


Dn_EtOH vs CS_EtOH
16
8
1.3
0
−log10(padj)
-5
0
5
log2(fold change)
Differential Expressed Genes (10)
up regulated: 4
down regulated: 6

D


Dn_EtOH vs CS_Naive
22
11
1.3
0
−log10(padj)
-8
-4
0
4
log2(fold change)
Differential Expressed Genes (73)
up regulated: 8
down regulated: 65

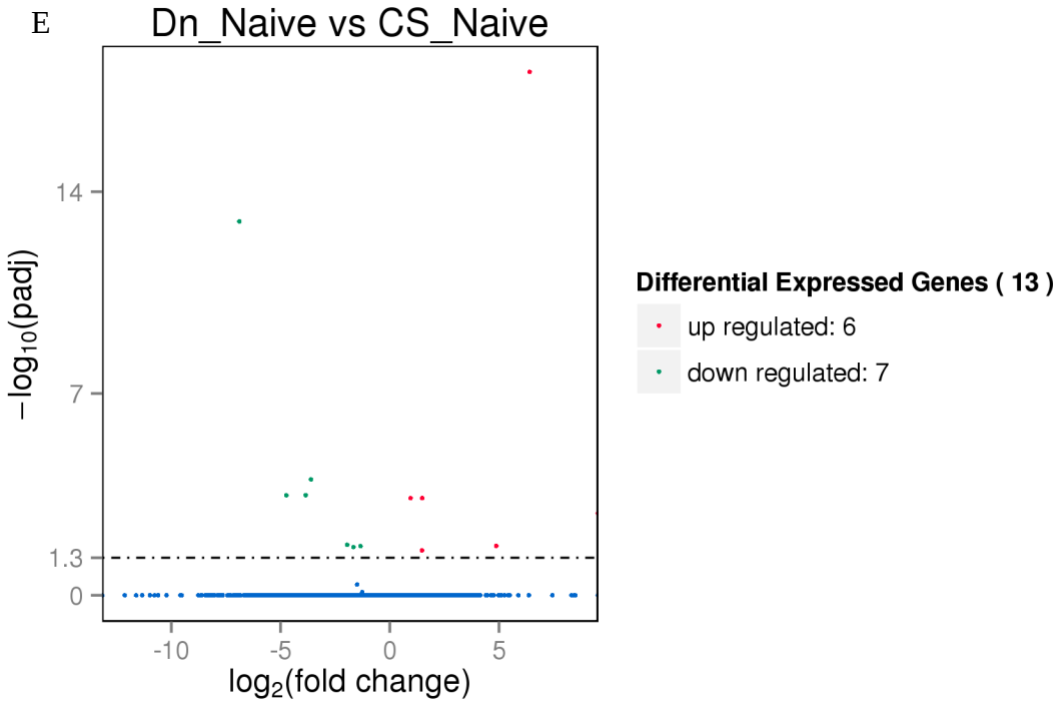


Figure 5. Volcano plots on the differential gene expression.
Volcano plots were generated to infer the overall distribution of differentially expressed genes. Since there were four replicates in each group, the DEseq eliminated the biological variation. The threshold for the elimination was the False Discovery Rate (FDR) adjusted *p*-value < 0.05. The vertical line shows -$\log_{10}$ of p adjusted value for each gene. Horizontal line is Log2-fold-change (Log2FC), which was calculated as log2FC=Log2(FPKM in CS_EtOH)-Log2(FPKM in CS_Naive). (A) CS_EtOH vs CS_Naive. 41 genes were up-regulated, and 99 genes were down-regulated CS_EtOH compared to CS_Naive. (B) CS_EtOH vs Dn_Naive. 1087 genes were up-regulated and 87 genes were down-regulated in CS_EtOH compared to Dn_Naive. (C) Dn_EtOH vs CS_EtOH. 4 genes were up-regulated and 6 genes were down-regulated in Dn_EtOH compared to CS_EtOH. (D) Dn_EtOH vs CS_Naive. 8 genes were up-regulated and 65 genes were down-regulated in Dn_EtOH compared to CS_Naive. (E) Dn_Naive vs Cs_Naive. 6 genes were up-regulated and 7 genes were down-regulated in Dn_Naive compared to Cs_Naive. CS_Naive: Wild type flies were exposed to 100% H_2O vapor for the same time as CS_EtOH group were exposed to ethanol vapor, and then kept in vials containing regular food for four hours at 25℃. CS_EtOH: Wild type flies were exposed to 50% ethanol vapor until 90% sedation, and then kept in vials containing regular food for four hours at 25℃. Dn_Naive: *Dunc13*^{P84200}/+ heterozygotes were exposed to 100% H_2O vapor for the same time as the Dn_EtOH group was exposed to ethanol vapor, and then kept in vials containing regular food for four hours at 25℃. Dn_EtOH: *Dunc13*^{P84200}/+ heterozygotes were exposed to 50% ethanol vapor until 90% sedation, and then kept in vials containing regular food for four hours at 25℃.

Gene_id	p-adjusted	up/ down-regulation	Gene name	Gene description
FBgn0004181	0.001	up	Ebp	Ejaculatory bulb protein [Source:FlyBase;Acc:FBgn0004181]
FBgn0004428	0.041	up	LysE	Lysozyme E [Source:FlyBase;Acc:FBgn0004428]
FBgn0011555	0.013	up	thetaTry	thetaTrypsin [Source:FlyBase;Acc:FBgn0011555]
FBgn0011694	0.007	up	EbpII	Ejaculatory bulb protein II [Source:FlyBase;Acc:FBgn0011694]
FBgn0015010	0.028	up	Ag5r	Antigen 5-related [Source:FlyBase;Acc:FBgn0015010]
FBgn0028583	0.027	up	lcs	la costa [Source:FlyBase;Acc:FBgn0028583]
FBgn0031176	0.010	up	whe	what else [Source:FlyBase;Acc:FBgn0031176]
FBgn0031277	0.018	up	CG13947	-
FBgn0032144	0.027	up	CG17633	-
FBgn0032505	0.005	up	CG16826	-
FBgn0032913	0.000	up	CG9259	-
FBgn0033774	0.001	up	CG12374	-
FBgn0034553	0.019	up	CG9993	-
FBgn0035358	0.039	up	CG14949	-
FBgn0035666	0.008	up	Jon65Aii	Jonah 65Aii [Source:FlyBase;Acc:FBgn0035666]
FBgn0035781	0.013	up	CG8560	-
FBgn0038481	0.017	up	CG17475	-
FBgn0038790	0.006	up	MtnC	Metallothionein C [Source:FlyBase;Acc:FBgn0038790]
FBgn0039311	0.004	up	CG10513	-
FBgn0039471	0.021	up	CG6295	-
FBgn0039472	0.007	up	CG17192	-
FBgn0039760	0.008	up	CG9682	-
FBgn0039787	0.029	up	CG9702	-
FBgn0040350	0.017	up	CG3690	-
FBgn0040531	0.041	up	CG11741	-
FBgn0043825	0.039	up	CG18284	-
FBgn0047334	0.020	up	BG642312	-
FBgn0051233	0.010	up	CG31233	-
FBgn0051259	0.002	up	CG31259	-
FBgn0051267	0.011	up	CG31267	-
FBgn0052669	0.029	up	CG32669	-
FBgn0083936	0.023	up	Acp54A1	Accessory gland protein 54A1 [Source:FlyBase;Acc:FBgn0083936]
FBgn0085241	0.030	up	CG34212	-
FBgn0250757	0.033	up	CG42235	-
FBgn0259951	0.045	up	Sfp24Ba	Seminal fluid protein 24Ba [Source:FlyBase;Acc:FBgn0259951]
FBgn0259968	0.021	up	Sfp60F	Seminal fluid protein 60F [Source:FlyBase;Acc:FBgn0259968]
FBgn0259975	0.004	up	Sfp87B	Seminal fluid protein 87B [Source:FlyBase;Acc:FBgn0259975]
FBgn0262547	0.027	up	CG43101	-
FBgn0263763	0.001	up	CG43680	-
FBgn0265264	0.045	up	CG17097	-
FBgn0266455	0.004	up	CG45080	-
FBgn0000277	0.021	down	CecA2	Cecropin A2 [Source:FlyBase;Acc:FBgn0000277]
FBgn0000278	0.030	down	CecB	Cecropin B [Source:FlyBase;Acc:FBgn0000278]
FBgn0000279	0.000	down	CecC	Cecropin C [Source:FlyBase;Acc:FBgn0000279]
FBgn0001224	0.001	down	Hsp23	Heat shock protein 23 [Source:FlyBase;Acc:FBgn0001224]
FBgn0001225	0.000	down	Hsp26	Heat shock protein 26 [Source:FlyBase;Acc:FBgn0001225]
FBgn0001230	0.002	down	Hsp68	Heat shock protein 68 [Source:FlyBase;Acc:FBgn0001230]
FBgn0001311	0.004	down	kkv	krotzkopf verkehrt [Source:FlyBase;Acc:FBgn0001311]
FBgn0002440	0.013	down	l(3)mbn	lethal (3) malignant blood neoplasm [Source:FlyBase;Acc:FBgn0002440]
FBgn0004577	0.002	down	Pxd	Peroxidase [Source:FlyBase;Acc:FBgn0004577]
FBgn0004777	0.027	down	Ccp84Ag	-
FBgn0004782	0.000	down	Ccp84Ab	-
FBgn0004783	0.001	down	Ccp84Aa	-
FBgn0005664	0.000	down	Crys	Crystallin [Source:FlyBase;Acc:FBgn0005664]
FBgn0012042	0.000	down	AttA	Attacin-A [Source:FlyBase;Acc:FBgn0012042]
FBgn0013279	0.004	down	Hsp70Bc	Heat-shock-protein-70Bc [Source:FlyBase;Acc:FBgn0013279]
FBgn0014454	0.000	down	Acp1	Adult cuticle protein 1 [Source:FlyBase;Acc:FBgn0014454]
FBgn0014859	0.004	down	Hr38	Hormone receptor-like in 38 [Source:FlyBase;Acc:FBgn0014859]
FBgn0023549	0.027	down	Mct1	Monocarboxylate transporter 1 [Source:FlyBase;Acc:FBgn0023549]
FBgn0024989	0.003	down	CG3777	-
FBgn0025835	0.001	down	CG17707	-
FBgn0026077	0.036	down	Gasp	-

FBgn0027600	0.010	down	obst-B	obstructor-B [Source:FlyBase;Acc:FBgn0027600]
FBgn0028544	0.000	down	CG16884	-
FBgn0028573	0.013	down	prc	pericardin [Source:FlyBase;Acc:FBgn0028573]
FBgn0028938	0.019	down	CG16886	-
FBgn0029573	0.043	down	CG14770	-
FBgn0030073	0.005	down	CG10962	-
FBgn0030114	0.000	down	CG17754	-
FBgn0030357	0.000	down	Sclp	-
FBgn0030398	0.000	down	Cpr11B	Cuticular protein 11B [Source:FlyBase;Acc:FBgn0030398]
FBgn0030562	0.027	down	CG9400	-
FBgn0030593	0.021	down	CG9512	-
FBgn0030829	0.001	down	CG12998	-
FBgn0030830	0.000	down	CG5172	-
FBgn0031692	0.033	down	TpnC25D	Troponin C at 25D [Source:FlyBase;Acc:FBgn0031692]
FBgn0031939	0.014	down	CG13796	-
FBgn0031940	0.005	down	CG7214	-
FBgn0031942	0.006	down	CG7203	-
FBgn0031971	0.010	down	Sirup	Starvation-upregulated protein [Source:FlyBase;Acc:FBgn0031971]
FBgn0032283	0.000	down	CG7296	-
FBgn0032538	0.004	down	CG16885	-
FBgn0032803	0.009	down	CG13082	-
FBgn0033308	0.001	down	CG8736	-
FBgn0033593	0.005	down	Listericin	-
FBgn0033667	0.038	down	reb	rebuf [Source:FlyBase;Acc:FBgn0033667]
FBgn0033668	0.018	down	exp	expansion [Source:FlyBase;Acc:FBgn0033668]
FBgn0033730	0.035	down	Cpr49Ag	Cuticular protein 49Ag [Source:FlyBase;Acc:FBgn0033730]
FBgn0033817	0.039	down	GstE14	Glutathione S transferase E14 [Source:FlyBase;Acc:FBgn0033817]
FBgn0033830	0.000	down	CG10814	-
FBgn0033869	0.034	down	Cpr50Cb	Cuticular protein 50Cb [Source:FlyBase;Acc:FBgn0033869]
FBgn0034253	0.038	down	CG10936	-
FBgn0034407	0.006	down	DptB	Diptericin B [Source:FlyBase;Acc:FBgn0034407]
FBgn0034511	0.017	down	GNBP-like3	GNBP-like 3 [Source:FlyBase;Acc:FBgn0034511]
FBgn0034709	0.001	down	Swim	Secreted Wg-interacting molecule [Source:FlyBase;Acc:FBgn0034709]
FBgn0034974	0.009	down	CG16786	-
FBgn0035173	0.039	down	CG13907	-
FBgn0035281	0.004	down	Cpr62Bc	Cuticular protein 62Bc [Source:FlyBase;Acc:FBgn0035281]
FBgn0035398	0.000	down	Cht7	Chitinase 7 [Source:FlyBase;Acc:FBgn0035398]
FBgn0035510	0.033	down	Cpr64Aa	Cuticular protein 64Aa [Source:FlyBase;Acc:FBgn0035510]
FBgn0035546	0.000	down	CG11345	-
FBgn0035806	0.038	down	PGRP-SD	Peptidoglycan recognition protein SD [Source:FlyBase;Acc:FBgn0035806]
FBgn0035844	0.003	down	CG13676	-
FBgn0036323	0.000	down	CG14118	-
FBgn0036585	0.000	down	CG13071	-
FBgn0036590	0.002	down	CG13065	-
FBgn0036600	0.000	down	CG13043	-
FBgn0036881	0.003	down	Cpr76Bd	Cuticular protein 76Bd [Source:FlyBase;Acc:FBgn0036881]
FBgn0036992	0.006	down	CG11796	-
FBgn0037933	0.011	down	Ho	Heme oxygenase [Source:FlyBase;Acc:FBgn0037933]
FBgn0038180	0.041	down	Cht5	Chitinase 5 [Source:FlyBase;Acc:FBgn0038180]
FBgn0038405	0.003	down	CG8927	-
FBgn0038511	0.002	down	cysu	Curly Su [Source:FlyBase;Acc:FBgn0038511]
FBgn0038720	0.006	down	CG6231	-
FBgn0039161	0.044	down	CG13606	-
FBgn0039324	0.003	down	CG10553	-
FBgn0039480	0.005	down	Cpr97Ea	Cuticular protein 97Ea [Source:FlyBase;Acc:FBgn0039480]
FBgn0039481	0.028	down	Cpr97Eb	Cuticular protein 97Eb [Source:FlyBase;Acc:FBgn0039481]
FBgn0039593	0.001	down	Sid	Stress induced DNase [Source:FlyBase;Acc:FBgn0039593]
FBgn0039805	0.005	down	Cpr100A	Cuticular protein 100A [Source:FlyBase;Acc:FBgn0039805]
FBgn0040343	0.028	down	CG3713	-
FBgn0040359	0.000	down	CG11380	-
FBgn0040637	0.000	down	CG11458	-
FBgn0040794	0.010	down	CG13056	-
FBgn0041579	0.000	down	AttC	Attacin-C [Source:FlyBase;Acc:FBgn0041579]
FBgn0041581	0.000	down	AttB	Attacin-B [Source:FlyBase;Acc:FBgn0041581]

FBgn0041581	0.000	down	AttB	Attacin-B [Source:FlyBase;Acc:FBgn0041581]
FBgn0042201	0.045	down	Nplp3	Neuropeptide-like precursor 3 [Source:FlyBase;Acc:FBgn0042201]
FBgn0046875	0.024	down	Obp83g	Odorant-binding protein 83g [Source:FlyBase;Acc:FBgn0046875]
FBgn0052499	0.025	down	Cda4	Chitin deacetylase-like 4 [Source:FlyBase;Acc:FBgn0052499]
FBgn0052865	0.031	down	alphagamma-el	-
FBgn0058198	0.001	down	CG40198	-
FBgn0082582	0.009	down	tmod	tropomodulin [Source:FlyBase;Acc:FBgn0082582]
FBgn0085356	0.000	down	CG34327	-
FBgn0086348	0.008	down	se	sepia [Source:FlyBase;Acc:FBgn0086348]
FBgn0260011	0.000	down	NimC4	Nimrod C4 [Source:FlyBase;Acc:FBgn0260011]
FBgn0260479	0.000	down	CG31904	-
FBgn0260653	0.006	down	serp	serpentine [Source:FlyBase;Acc:FBgn0260653]
FBgn0261341	0.006	down	verm	vermiform [Source:FlyBase;Acc:FBgn0261341]
FBgn0263132	0.007	down	Cht6	Chitinase 6 [Source:FlyBase;Acc:FBgn0263132]
FBgn0265356	0.021	down	tn	thin [Source:FlyBase;Acc:FBgn0265356]

Table 1. The Different Expression Gene List between naive wild type and pre-exposed wild type. The genes differentially expressed between naive wild type and pre-exposed wild type are listed. The read count value from the gene expression level analysis was analyzed by DESeq (Anders et al, 2010). The direction of gene regulation (up/down) in the pre-exposed wild type is indicated in the third column.

3. Analysis by DEseq2, Limma-voom, and edgeR

The read-counts file containing sequencing data has been further analyzed using 3 differential expression analysis (DE analysis) tools mainly to cross-validate our results across multiple programs. We have used: edgeR 2 (Figure 6), Limma-Voom (Figure 7), and DEseq2 (Figure 8) from https://gallery.shinyapps.io/DEApp/, which is developed by the bioinformatics core, Center for Research Informatics (CRI), University of Chicago. Finally, we generated a Venn diagram that shows the overlapped genes that were differentially expressed in all three tools (Figure 9). This application uses the open-source R packages of the three programs. Below are the results obtained from these analyses.

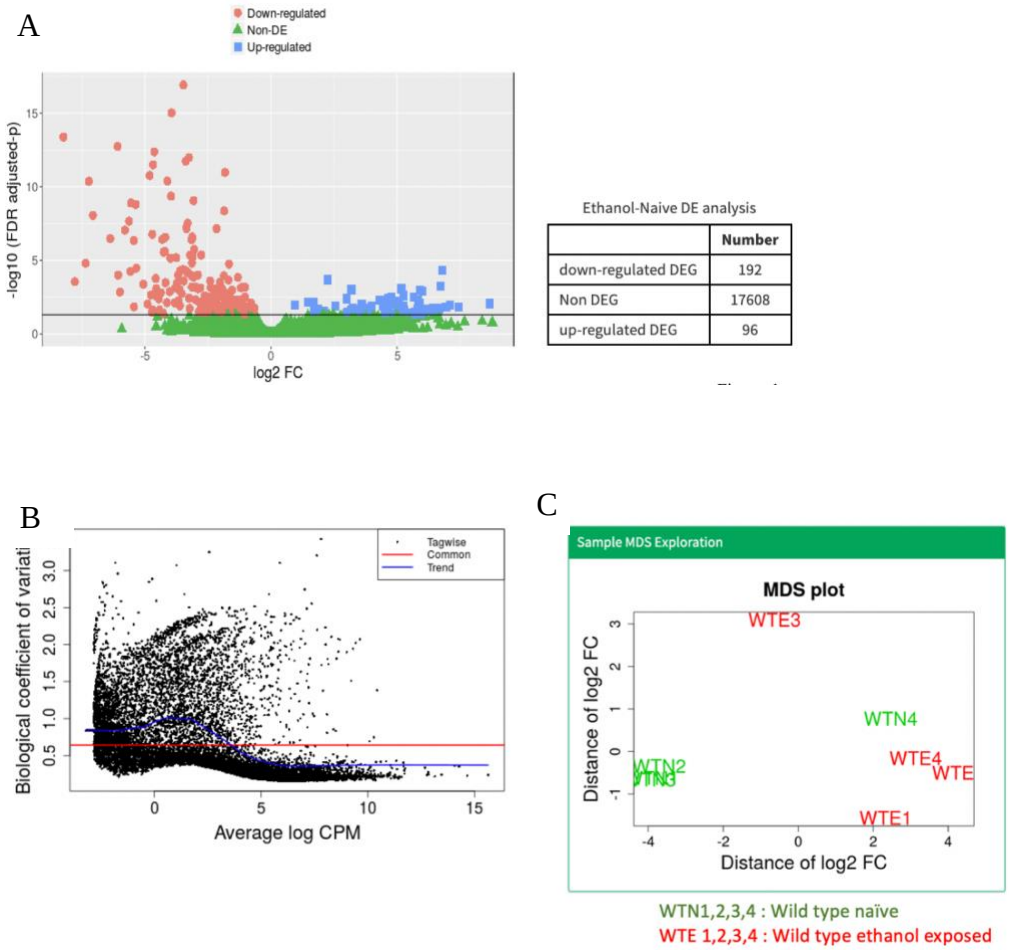


Figure 6. The results of edgeR analysis. This tool is built based on negative binomial distributions and implements a range of statistical methodology including empirical Bayes estimation, exact tests, generalized linear models, and quasi-likelihood tests. Figure 6A s' the volcano diagram and differential expression analysis. Log2-fold-change (Log2FC) w... calculated as log2FC = Log2 (gene A expression)-Log2 (gene B expression). For the gene expression, fragments per kilobase of exon model per million read mapped (FPKM) was used. The number of reads of the particular gene was counted by considering the gene length. False Discovery Rate (FDR) is the rate to make type I error in the null hypothesis test. FDR adjusted *p*-value was 0.050. The result showed that 192 genes were down-regulated, and 96 genes were up-regulated. Figure 6B is the biological coefficient of variation (BCV) diagram, which indicates the gene's dispersion among replicates. The number indicates the percentage of difference in gene expression between samples. Counts per million (CPM) indicate depth-normalized counts. The number of the gene is different among the samples. Therefore, the gene expression was divided by the total counts of the sample and multiplied by one million. The trend line (blue) is calculated from tagwise (black) to show the trend of variation. Our trend shows BCV is high for the genes that were expressed in a small amount. In addition, this figure shows that gene expression that is plotted in low average log CPM and high level of BCV can be interpreted as noise since the variation is high among the samples and the level of gene expression is low. Multi-Dimensional Scaling (MDS) shows the level of similarity among the samples (Figure 6C). The algorithm processed each gene expression into the dimensions.

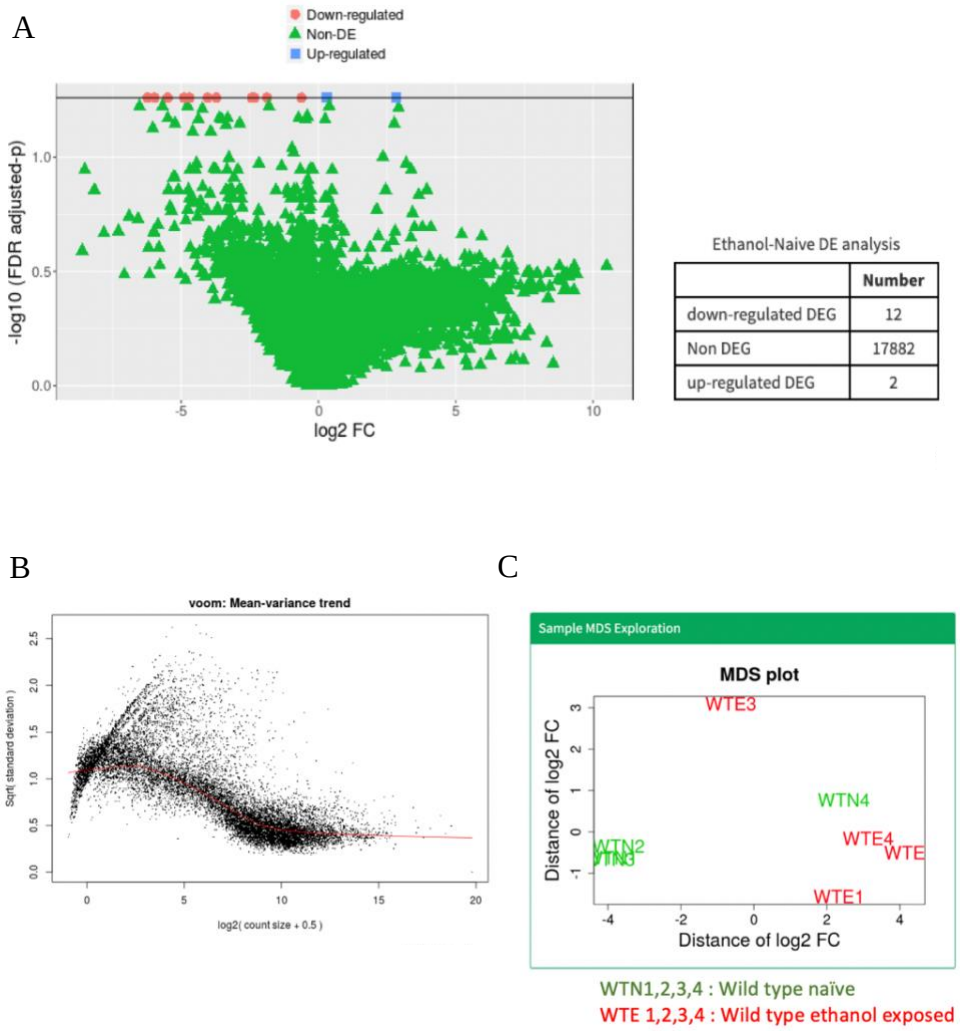


Figure 7. The results of Limma-Voom. This tool estimates the mean-variance relationship of the log-counts, generates a precision weight for each observation and enters these into the limma empirical Bayes analysis pipeline. Sharp decreasing trends indicate low biological variation. For DE analysis in limma-voom, we used FDR adjusted *p*-value of 0.055 instead of 0.050 since no differential expression was detected with 0.050, in other words, the lowest voom adjusted *p*-value was 0.05496. As Figure 7A, the result was that 12 genes were down-regulated and 2 genes were up-regulated. Figure 7B shows a mean-variance trend. Trend line (red) that was calculated based on the square root of the standard deviation of FPKM. Multi-Dimensional Scaling (MDS) shows the level of similarity among the samples (Figure 7C). The algorithm processed each gene expression into the dimensions.

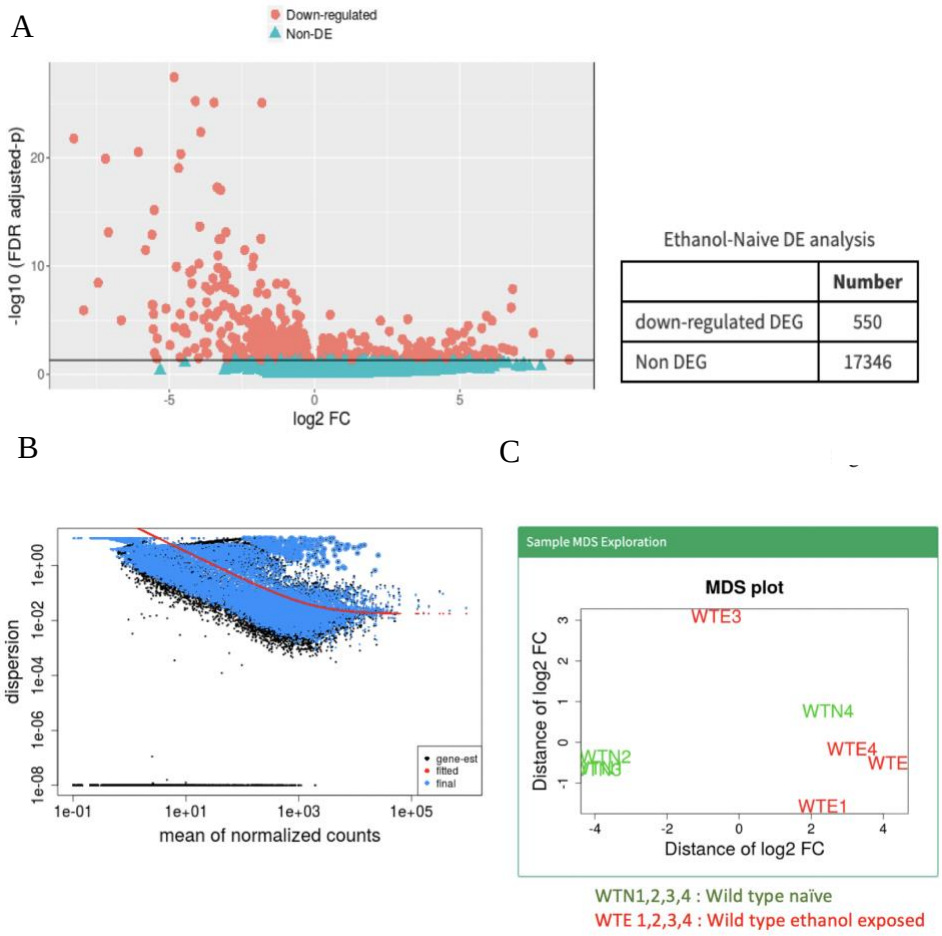


Figure 8. The results of DEseq2 analysis. DEseq2 is based on a negative binomial distribution model. This tool estimates variance-mean dependence in count data from high-throughput sequencing assays and tests for differential expression. As Figure 8A, we used the FDR adjusted *p*-value of 0.050. The result was that 550 genes were down-regulated and zero genes were up-regulated. Figure 8B shows a gene-wise dispersion. Final values (blue) are calculated from the fitted value (red) that is calculated from gene-est (black) on an empirical Bayes approach. Multi-Dimensional Scaling (MDS) shows the level of similarity among the samples (Figure 8C). The algorithm processed each gene expression into the dimensions.

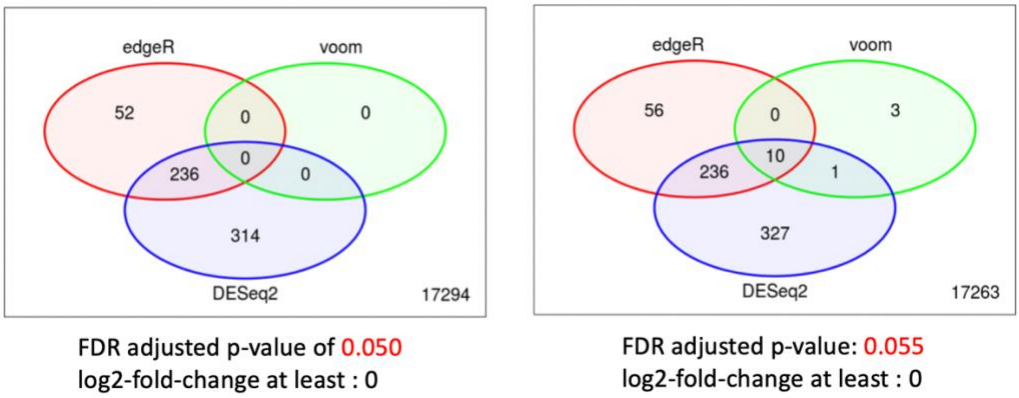


Figure 9. Venn diagram of among the results from edgeR, DEseq2, and Limma-voom. Overlapped genes that were differentially expressed in wild type *Drosophila* after ethanol exposure in three different analyses were shown in the Venn diagram. The ten overlapped gene names, gene descriptions, and regulation directions are described in table 3. When the FDR adjusted *p*-value of 0.050 was used, there were no overlapping genes among three tools, since, in Limma (left), there was no differential expression. Therefore, instead, we used the FDR adjusted *p*-value of 0.055 (right). We detected 10 overlapping genes that are differentially expressed. Of those genes, 9 genes were downregulated, and 1 gene was upregulated after ethanol exposure (Table 2).

Since all of these three DE analysis tools were built and based on different algorithms and different methods of normalization, the results from each of these tools were different.

To conclude the results from all these 3 platforms, a Venn diagram overlapping genes was plotted to find the common genes which are differentially expressed across 3 tools (Figure 9). From the analysis, we found that there are 10 overlapping genes that are differentially expressed across 3 different platforms. As shown below, there are 9 genes that are downregulated, and 1 gene is being upregulated when ethanol is administered (Table 2). The 10 overlapping genes from this study were not overlapped with the 29 overlapping genes from the multi-study comparison among the studies from Kong, E. C., et al, Nancy L. Urizar, et al, and Morozova, T. V., et al,. (Figure 10) As explained above, they used different time periods of ethanol exposure time and incubation time after ethanol exposure, which could account for the lack of overlapping genes identified between these studies.

Gene ID	up/ down - regulation	Gene name	Gene description
FBgn0040637	down	-	Insufficient genetic data for FlyBase to solicit a summary. [Date last reviewed: 2016-06-30]
FBgn0032283	down	-	Insufficient genetic data for FlyBase to solicit a summary. [Date last reviewed: 2016-06-30]
FBgn0012042	down	Attacin-A	Attacin-A (AttA) encodes an antibacterial peptide with activity against Gram-negative bacteria. It is expressed in the fat body and various epithelia under the regulation of the immune deficiency and, to a lesser extent, Toll pathways. [Date last reviewed: 2019-03-07]
FBgn0035548	down	-	Insufficient genetic data for FlyBase to solicit a summary. [Date last reviewed: 2016-06-30]
FBgn0034828	down	-	Insufficient genetic data for FlyBase to solicit a summary. [Date last reviewed: 2016-10-13]
FBgn0002440	down	-	lethal (3) malignant blood neoplasm (l(3)mbn) is a tumor suppressor gene with mutants showing abnormal proliferation of blood cells, differentiation of lamellocytes and melanotic nodule formation in the larva. [Date last reviewed: 2019-09-26]
FBgn0000279	down	-	Cecropin C (CecC) encodes an antibacterial peptide with activity against Gram-negative bacteria. It is strongly expressed at the pupal stage. [Date last reviewed: 2019-03-07]
FBgn0000278	down	-	Cecropin B (CecB) encodes an helicoidal antibacterial peptide with activity against Gram-negative bacteria. It is mostly expressed at the pupal stage. [Date last reviewed: 2019-03-07]
FBgn0033720	up	-	Insufficient genetic data for FlyBase to solicit a summary. [Date last reviewed: 2019-03-07]
FBgn0014018	down	-	Relish (Rel) encodes a transcription factor and the downstream component of the immune deficiency pathway, which regulates the antibacterial response and other less characterized cellular processes. [Date last reviewed: 2019-03-14]

Table 2. The list of the overlapped genes differentially expressed in edgeR, DEseq2, and Limma-voom. 10 genes overlapped in edgeR, DEseq2, and Limma-voom, as shown in figure 7, are shown with their gene name, the direction of differential expression, and the gene description. The direction of gene regulation (up/down) in the pre-exposed wild type was indicated in the second column.

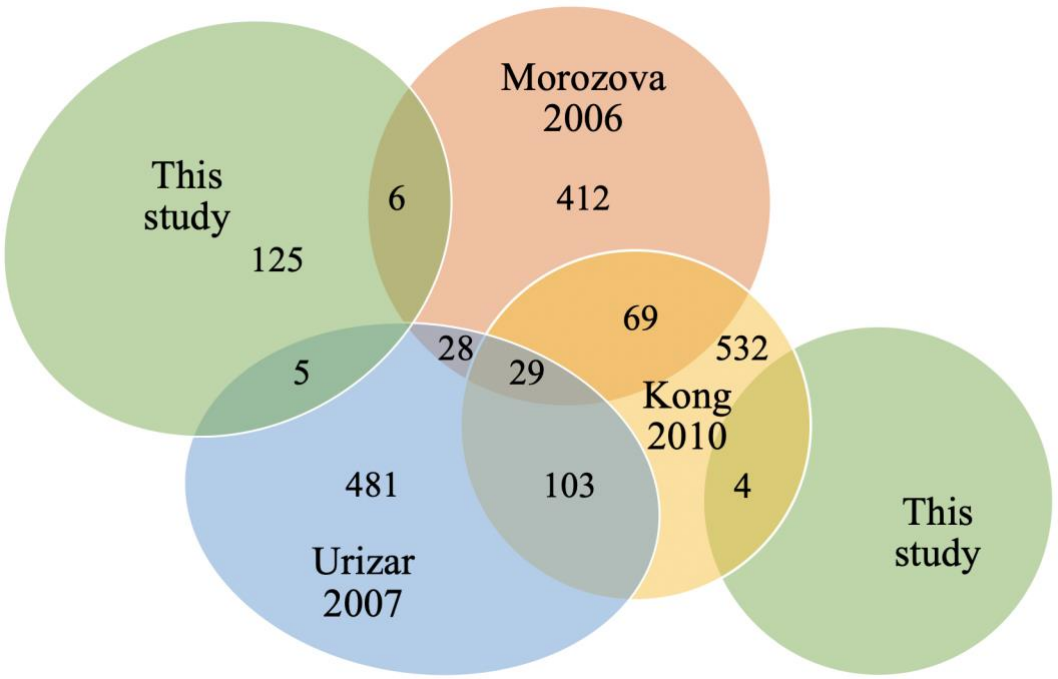
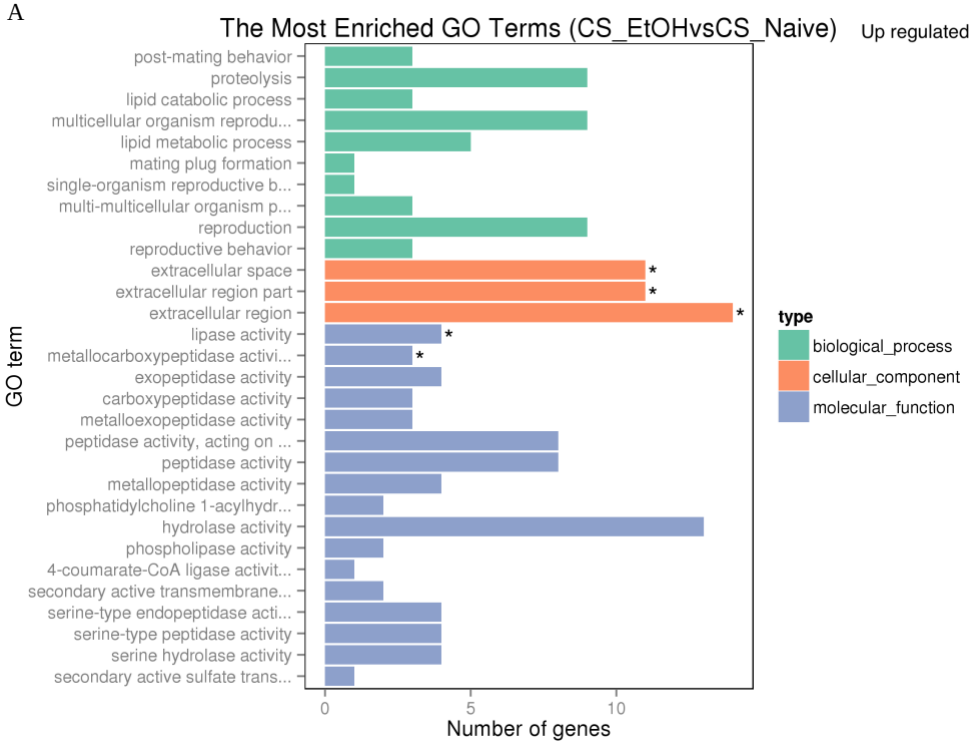


Figure 10. Venn diagram on gene expression overlapped among this study and previous studies. Genes that were differentially expressed in wild type *Drosophila* after ethanol exposure in three previous microarray studies and in this RNA-seq study were compared. The gene names, gene descriptions, and regulation directions are described in table 4 (Kong, Allouche, et al., 2010; Morozova, Anholt, & Mackay, 2006, 2007; Nancy L. Urizar, 2007). For this Venn diagram, 140 genes differentially expressed in pre-exposed wild type compared to naive wild type (table 1) were used.

4. GO terms analysis

To investigate the response of the genes that were differentially expressed in DE analysis, I analyzed the gene ontology of the differentially expressed genes (GO) (Figure 11). Gene ontology (GO) terms describe the role of a gene product with respect to three aspects; molecular function, cellular component, and biological process (Ashburner et al., 2000; The Gene Ontology Consortium, 2018). Based on GO terms analysis, I found the three pathways; serine related pathways, lipid-related pathways, and carboxypeptidase related pathways could be possibly involved in ethanol tolerance formation. Those pathways are discussed in the discussion section.

A


The Most Enriched GO Terms (CS_EtOHvsCS_Naive) Up regulated
post-mating behavior
proteolysis
lipid catabolic process
multicellular organism reprodu...
lipid metabolic process
mating plug formation
single-organism reproductive b...
multi-multicellular organism p...
reproduction
reproductive behavior
extracellular space
extracellular region part
extracellular region
lipase activity
metallocarboxypeptidase activi...
exopeptidase activity
carboxypeptidase activity
metalloexopeptidase activity
peptidase activity, acting on ...
peptidase activity
metallopeptidase activity
phosphatidylcholine 1-acylhydr...
hydrolase activity
phospholipase activity
4-coumarate-CoA ligase activit...
secondary active transmembrane...
serine-type endopeptidase acti...
serine-type peptidase activity
serine hydrolase activity
secondary active sulfate trans...
GO term
0
5
10
Number of genes
type
biological_process
cellular_component
molecular_function

B

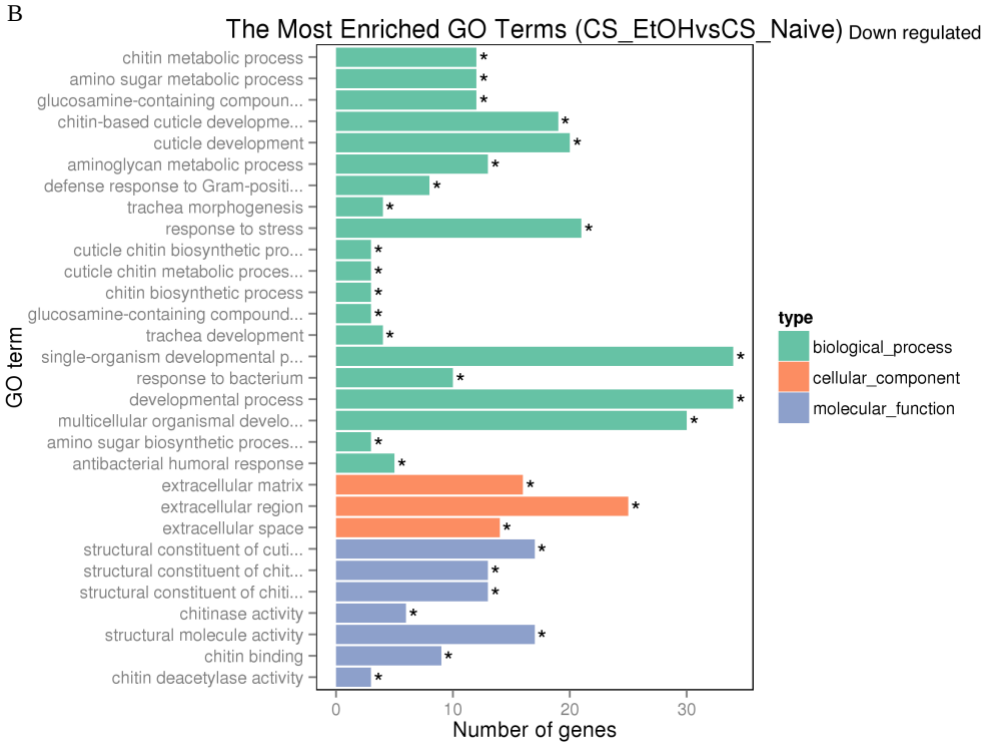

The Most Enriched GO Terms (CS_EtOHvsCS_Naive) Down regulated
chitin metabolic process
amino sugar metabolic process
glucosamine-containing compoun...
chitin-based cuticle developme...
cuticle development
aminoglycan metabolic process
defense response to Gram-positi...
trachea morphogenesis
response to stress
cuticle chitin biosynthetic pro...
cuticle chitin metabolic proces...
chitin biosynthetic process
glucosamine-containing compound...
trachea development
single-organism developmental p...
response to bacterium
developmental process
multicellular organismal develo...
amino sugar biosynthetic proces...
antibacterial humoral response
extracellular matrix
extracellular region
extracellular space
structural constituent of cuti...
structural constituent of chit...
structural constituent of chiti...
chitinase activity
structural molecule activity
chitin binding
chitin deacetylase activity
GO term
0
10
20
30
Number of genes
type
biological_process
cellular_component
molecular_function

Figure 11. GO Enrichment Bar Chart of DEGs. This chart shows the differentially expressed genes enriched GO terms and counts of genes for each GO terms. The x-axis first containing the number of genes that are categorized in GO terms. The y-axis shows the GO terms enriched. Different colors were used to differentiate biological process, cellular component, and molecular function. (*) indicates the most enriched GO terms. (A) 42 genes were up-regulated in 5 GO terms between CS_EtOH vs CS_Naive. (B) 386 genes were down-regulated in 30 most enriched GO terms between CS_EtOH vs CS_Naive.
CS_Naive: Wild type flies were exposed to 100% H_2O vapor for the same time as CS_EtOH group were exposed to ethanol vapor, and then kept in vials containing regular food for four hours at 25℃. CS_EtOH: Wild type flies were exposed to 50% ethanol vapor until 90% sedation, and then kept in vials containing regular food for four hours at 25℃.

(i) Serine related pathway

According to the GO enrichment analysis between the naive wild type and ethanol-exposed wild type, the genes that are responsible for serine-type endopeptidase activity, serine-type peptidase activity, and serine hydrolase activity, were significantly upregulated (Figure 11). According to the study from Kong et al., 2010, when the CG3011 locus, which encodes an enzyme that metabolizes glycine to serine, was disrupted, ethanol-induced hyperactivity increased; in addition, there was positive correlation between the sedation tolerance and the distance of activity after ethanol exposure (Kong, Allouche, et al., 2010). Although the relationship between the genes we identified that impact serine recognition and metabolism may be coincidental, there may also be an underlying relationship that is currently unknown, perhaps related to the shared chemistry of ethanol and serine.

(ii) Lipid related pathway

Alcohol consumption increases fat in the liver by esterifying the fatty acid to triglycerides, phospholipids, and cholesterol esters (Baraona & Lieber, 1979). The GO enrichment analysis showed the 14 genes were differentially expressed in the lipid-related GO terms, which are lipid catabolic processes, lipid metabolic processes, lipase activity, and

phospholipase activity (Figure 11). This result suggests that ethanol exposure may induce lipid accumulation in *Drosophila* through esterification, which may then cause the upregulation of lipid-related genes as a homeostatic response.

(iii) Carboxypeptidase related pathway

We identified 6 genes related to carboxypeptidase activity that were significantly upregulated (Figure 11). It was previously shown that alcohol preference decreased in mice after they were treated with the carboxypeptidase inhibitors hydrocinnamic acid and D-phenylalanine (Blum, Briggs, Trachtenberg, Delallo, & Wallace, 1987). Since alcohol preference is positively correlated to the level of ethanol tolerance, it is possible that inhibiting carboxypeptidase activity decreases tolerance formation (Devineni et al., 2011).

CHAPTER IV.

DISCUSSION

1. *Dunc13*$_{P84200}$/+ heterozygotes significantly decreased alcohol sensitivity and increased alcohol tolerance.

I found that *Dunc13*$_{P84200}$/+ heterozygotes that did not show sedative-like effect due to the low level of *Dunc13* in naive condition demonstrate increased resistance to the sedative effect of ethanol compared to wild type flies (Figure 1), which is consistent with previous work (Xu et. al., 2018). Interestingly, these *Dunc13*$_{P84200}$/+ heterozygotes are also resistant to the inhibitory effects of ethanol on synaptic vesicle release (S. Xu et al., 2018). *Dunc13*$_{P84200}$/+ heterozygotes have chronically low levels of *Dunc13* activity due to the presence of a single loss-of-function mutation. This result suggested the hypothesis that chronically reduced *Dunc13* activity-induced homeostatic response to compensate for decreased activity of *Dunc13*, which mimics or phenocopies the effects of chronic exposure to ethanol. As a result, *Dunc13*$_{P84200}$/+ heterozygotes had a higher level of ethanol tolerance compared to naive wild type. Table 3 shows the result of differential gene expression between naive wild type and naive *Dunc13*$_{P84200}$/+ heterozygotes. Although the gene that was possibly related to the role of Dunc13 in neurotransmitter release was not found in the differential gene expression between naive wild type and naive *Dunc13*$_{P84200}$/+ heterozygotes, those genes in table 3 could play the role of inducing ethanol tolerance in the flies that were chronically reduced *Dunc13* activity. To investigate if the ethanol tolerance formation by genetically reduced *Dunc13* activity mimics the

ethanol tolerance formation by alcohol exposure, differential gene expression level analysis between *Dunc13*^{P84200}/+ heterozygotes and wild type that chronic tolerance was induced is required.

Gene ID	p-adjusted	up/ down- regulation	Gene name	Gene description
FBgn0003308	0.0279	up	ry	rosy [Source:FlyBase;Acc:FBgn0003308]
FBgn0015714	0.0000	up	Cyp6a17	Cytochrome P450-6a17 [Source:FlyBase;Acc:FBgn0015714]
FBgn0030773	0.0004	up	CG9676	-
FBgn0052523	0.0004	up	CG32523	-
Novel00027	0.0195	up	--	envelope protein [Drosophila melanogaster]
Novel00140	0.0014	up	--	GH15551p [Drosophila melanogaster]
FBgn0033395	0.0001	down	Cyp4p2	-
FBgn0041581	0.0212	down	AttB	Attacin-B [Source:FlyBase;Acc:FBgn0041581]
FBgn0051683	0.0179	down	CG31683	-
FBgn0053503	0.0003	down	Cyp12d1-d	-
FBgn0261675	0.0000	down	Npc1b	Niemann-Pick type C-1b [Source:FlyBase;Acc:FBgn0261675]
Novel00003	0.0003	down	--	gag-like polyprotein - fruit fly (Drosophila melanogaster) transposon HeT-A 17B3 (fragment)>gi|443791|emb|CAA54342.1| gag-like polypeptide [Drosophila melanogaster]
Novel00012	0.0199	down	--	RNA-directed DNA polymerase [Drosophila melanogaster]

Table 3. The Different Expression Gene List between naive wild type and naive *Dunc13*^{P84200}/+ heterozygotes.
The genes differentially expressed between naive wild type and naive *Dunc13*^{P84200}/+ heterozygotes. The read count value from the gene expression level analysis was analyzed by DESeq (Anders et al, 2010). The direction of gene regulation (up/down) in naive *Dunc13*^{P84200}/+ heterozygotes compared to naive wild type was indicated in the third column.

It was predicted that genetically reduced *Dunc13* activity by *Dunc13*^{P84200}/+ heterozygotes mimicked the ethanol tolerance formation induced by homeostatic response caused by reduced *Dunc13* activity by ethanol exposure. To examine the prediction, the different expression gene list between naive wild type and pre-exposed wild type (Table 1) and the one between naive wild type and naive *Dunc13*^{P84200}/+ heterozygotes (Table 3) was compared to check if there are genes that were differentially expressed in both gene list. There was no common gene that was differentially expressed in both lists. Although the result suggests that the genes responsible for the homeostatic response caused by *Dunc13*^{P84200}/+ heterozygotes and

caused by ethanol exposure were completely different, it is also possible that we are not looking a the appropriate time to find those differences, e.g., the genes that are responsible for chronic tolerance and the genes that are induced during rapid tolerance are likely different.

This study	up/ down	Morozova 2006	up/ down	Urizar 2007	up/ down	Kong 2010	up/ down	gene name	gene description
FBgn0032913	up	FBgn0032913	down	-	-	-	-	-	-
FBgn0035781	up	FBgn0035781	up	-	-	-	-	-	-
FBgn0038790	up	FBgn0038790	up	-	-	-	-	*MtnC*	Metallothionein C [Source:FlyBase;Acc:FBgn0038790]
FBgn0039311	up	-	-	FBgn0039311	down (acute) down (chronic)	-	-	-	-
FBgn0039760	up	FBgn0039760	down	-	-	-	-	-	-
FBgn0040531	up	-	-	FBgn0040531	down (acute) down (chronic)	-	-	-	-
FBgn0051259	up	FBgn0051259	down	-	-	-	-	-	-
FBgn0001225	down	-	-	-	-	FBgn0001225	down	*Hsp26*	Heat shock protein 26 [Source:FlyBase;Acc:FBgn0001225]
FBgn0005664	down	-	-	FBgn0005664	down	-	-	*Crys*	Crystallin [Source:FlyBase;Acc:FBgn0005664]
FBgn0014454	down	-	-	FBgn0014454	down	-	-	*Acp1*	Adult cuticle protein 1 [Source:FlyBase;Acc:FBgn0014454]
FBgn0001224	down	-	-	-	-	FBgn0001224	up	*Hsp23*	Heat shock protein 23 [Source:FlyBase;Acc:FBgn0001224]
FBgn0001230	down	-	-	-	-	FBgn0001230	up	*Hsp68*	Heat shock protein 68 [Source:FlyBase;Acc:FBgn0001230]
FBgn0014859	down	FBgn0014859	up	-	-	-	-	*Hr38*	Hormone receptor-like in 38 [Source:FlyBase;Acc:FBgn0014859]
FBgn0013279	down	-	-	-	-	FBgn0013279	up	*Hsp70Bc*	Heat-shock-protein-70Bc [Source:FlyBase;Acc:FBgn0013279]
FBgn0026077	down	-	-	FBgn0026077	down	-	-	*Gasp*	-

Table 4. The summary of gene expression overlapped among this study and previous studies. Genes that were differentially expressed in *Drosophila* after ethanol exposure in three previous microarray studies and in this RNA-seq study were listed. (Kong, Allouche, et al., 2010; Morozova et al., 2006, 2007; Nancy L. Urizar, 2007)

2. *Dunc13* mutant significantly impacted the transcriptional response to ethanol exposure

There were remarkable effects of *Dunc13*$_{P84200}$/+ heterozygotes on the gene expression. Although 140 genes were differentially expressed 4 hours after ethanol exposure in wild type flies (Figure 5A), no genes were differentially expressed 4 hours after ethanol exposure in *Dunc13*$_{P84200}$/+ heterozygotes. This result suggested that *Dunc13*$_{P84200}$/+ heterozygotes were transcriptionally resistant to the effects of the ethanol.

The expression of *slowpoke* that is required for ethanol tolerance formation (R. B. Cowmeadow et al., 2005; A. Ghezzi et al., 2013) was not up-regulated 4 hours after ethanol exposure in both wild type flies and *Dunc13*$_{P84200}$/+ heterozygotes in this study. The possible reason was that our differential expression was measured 4 hours after 1$_{st}$ ethanol exposure while the study from Ghezzi et. al., 2013 showed that *slowpoke was induced* 24 hours after 1$_{st}$ ethanol exposure (A. Ghezzi et al., 2013).

Rapid tolerance is protein synthesis independent, in other words, changes in the gene expression are not necessary for rapid tolerance (Atkinson, 2009; Berger et al., 2004). In this study, the differential gene expression analysis showed that there were gene expression changes in rapid tolerance, but they are not required for rapid tolerance. Gene expression that induces protein synthesis chronically is required for chronic tolerance (Atkinson, 2009; R. B. Cowmeadow et al., 2005).

3. Possible genes that are required for ethanol tolerance formation

In the following section, genes that are necessary for ethanol tolerance formation based on the results of the differential expression analysis are described (Figure 2 and Table 1).

(i) Immune response-related gene

The expression of *AttA, AttB, and AttC*, which express Attacin A, Attacin B, and Attacin C respectively were significantly decreased in Canton-S wild type flies that were exposed to ethanol vapor compared to naive Canton-S wild type flies (*AttA*; $p < 0.01$, *AttB*; $p < 0.01$, *AttC*; $p < 0.01$, Table 1). The role of Attacin is an immune effector molecule that prevents gram-negative bacteria from growing (J. Wang et al., 2008). *CecA2*, *CecB*, and *CecC*, which express Cecropin A2, Cecropin B, and Cecropin C, were also down-regulated after Canton-S wild type flies were exposed to ethanol vapor compared to naive Canton-S wild type flies *(CecA2*; $p < 0.05$, *CecB*; $p < 0.05$, *CecC*; $p < 0.01$, Table 1). The cecropins have roles of the innate immune response (Diamond, Beckloff, Weinberg, & Kisich, 2009). *PGRP-SD* was also down-regulated in Canton-S wild type flies after ethanol exposure (*PGRP-SD*; $p < 0.05$, Table 1). It expresses peptidoglycan recognition protein SD, and the role of this protein is to bind to the peptidoglycan of gram-positive bacteria to activate the toll pathway (Filipe, Tomasz, & Ligoxygakis, 2005; L. Wang et al., 2008). *Nimrod C4* that expresses an adult cuticle protein 65As was down-regulated in Canton-S wild type flies after ethanol exposure (*Nimrod C4; p* < 0.01, Table 1). This protein is a transmembrane phagocytic receptor that binds phosphatidylserine exposed on apoptotic cells (Kurucz et al., 2007). Since the role of the receptor is to be recognized and engulfed by an apoptotic cell during development, it plays an important role in the immune response. Phagocytosis is one of the most rapidly induced responses to microbial infection; therefore, it plays an important role in host defense (Midega et al., 2013). *Cht 7* that expresses chitinase 7was down-regulated in Canton-S wild type flies after ethanol exposure (*Cht 7; p* < 0.01, Table 1). Chitinase is an enzyme that hydrolyzes chitin (Hamid et al., 2013). Chitin is one of the elements of exoskeletal in *Drosophila* (Moussian, Schwarz, Bartoszewski, & Nüsslein-Volhard, 2005).

Chitinase is used when the organism needs to reshape its own chitin (Merzendorfer & Zimoch, 2003). It also has a role in the defense against fungal infection since chitin hydrolyzes the cell wall of fungi (Pusztahelyi, 2018).

The possible explanation that ethanol exposure caused gene regulation is that alcohol consumption induces the production of reactive acetaldehyde that causes oxidative stress (Bondy, 1992; Wu & Cederbaum, 2003), which initiates immune response such as releasing cytokine and inducing cell damages (Crapo, 2003). Therefore, immune response-related genes were up or down-regulated after ethanol exposure as early response gene expression.

(ii) Crystalline is involved in alcohol metabolism

The *Crys* gene, that expresses crystallin, was significantly down-regulated in Canton-S wild type flies after ethanol exposure (*Crys;* $p < 0.01$, Table 1). Crystallin is a predominant water-soluble protein found in the lens and cornea of the eye and forms the transparent structures within these tissues (Andley, 2007; Jester, 2008). Crystallin has been shown to catalyze the detoxification of alcohol-derived acetaldehyde and to metabolize corticosteroids, biogenic amines, neurotransmitters, and lipid peroxidation (Estey, Piatigorsky, Lassen, & Vasiliou, 2007; Lassen et al., 2006; Manzer et al., 2003). Interestingly, Crystallin alpha B was up-regulated in human alcoholics (Iwamoto et al., 2004). This study suggested that Crystallin plays an important role in alcohol metabolism, and its genes are regulated during alcohol consumption in humans.

(iii) Alcohol-related *Hr38* gene

Hr38 is the mammalian homolog of Nra1, Nr4a2, and Nr4a3 gene family, and is a *Drosophila* immediate-early response gene (Adhikari, Orozco, Randhawa, & Wolf, 2019; Chen,

Rahman, Guo, & Rosbash, 2016; Fujita et al., 2013). Previously, it was shown that the expression of Hr38 was increased 60 minutes after 30 minutes of 55% ethanol exposure (Adhikari et al., 2019). Furthermore, ethanol tolerance of *Hr38* null mutant heterozygotes was significantly lower, and overexpression of *Hr38* significantly increased ethanol tolerance (Adhikari et al., 2019). Since Hr38 is an immediate-early response gene, the fold change expression was a peak at 1 hour after ethanol exposure and returned to a normal level of fold change expression after the peak (Adhikari et al., 2019). Our differential gene expression analysis showed that fold change expression was significantly decreased 4 hours after the ethanol exposure in Canton-S wild type flies (*Hr38*; $p < 0.01$, Table 1).

(iv) Oxidative stress-related genes

Sid gene encodes the stress-induced DNase (SID), which was shown to protect the cell from oxidative damage (Seong et al., 2014). Oxidative stress is induced by the consumption of alcohol (Galicia-Moreno & Gutiérrez-Reyes, 2014). For example, ethanol is oxidized by alcohol dehydrogenase to acetaldehyde, while the cofactor NAD is reduced to NADH (Badawy, 1977a). NADH is used in the respiratory chain, which produces reactive oxidative species (Murphy, 2009). Furthermore, free radicals that cause oxidative stress are produced when acetaldehyde reacts with protein and lipids (Wu & Cederbaum, 2003). Previously, it was shown that the expression of *Sid* was significantly increased after the bacterial infection and the treatment to induce oxidative stress (Seong et al., 2014). In our study, the expression of *Sid* was significantly decreased after ethanol exposure in Canton-S wild type flies (*Sid*; $p < 0.01$, Table 1). The previous study and our study suggested that *Sid* was differentially expressed after ethanol exposure as a defense mechanism against oxidative stress caused by ethanol exposure. In

addition, the expression of *Pxd* was also down-regulated after the ethanol exposure in Canton-S wild type flies (*Pxd*; $p < 0.01$, Table 1). It was shown that *Pxd* also expresses peroxidase in response to oxidative stress (Logan-Garbisch et al., 2014). The *Cysu* gene, that expresses Curly Su enzyme, was shown to acts as a ROS-producing enzyme (Hurd, Liang, & Lehmann, 2015). In this study, *Cysu* was down-regulated in Canton-S wild type flies after ethanol exposure (*Cysu*; $p < 0.01$, Table 1). *Ho* gene that expresses heme oxygenase was also down-regulated in Canton-S wild type flies after ethanol exposure (*Ho*; $p < 0.05$, Table 1). Heme oxygenase is protective protein from oxidative stress by maintaining the homeostasis between productions of antioxidant and oxidant by oxidative cleaving of heme groups to produce carbon monoxide and Fe^{2+} (Araujo, Zhang, & Yin, 2012; Le, Xie, & Appel, 1999). Up-regulation of *Ho* gene was expected since ethanol consumption induces oxidative stress (Galicia-Moreno & Gutiérrez-Reyes, 2014). On the other hand, *Ho* gene was significantly down regulated. The possible explanation was that the up regulation of *Ho* gene stress by transcriptional changer against oxidative stress required a longer period of ethanol exposure that induces chronic exposure.

(v) The effect of alcohol consumption on carbohydrate metabolism

Chitin deacetylase-like 4 (*Cda4*) was down-regulated in Canton-S wild type flies after ethanol exposure (*Cda4*; $p < 0.05$, Table 1). *Cda4* is involved in the metabolism of carbohydrates by removing acetyl groups to produce chitosan from chitin (Tsigos, Martinou, Kafetzopoulos, & Bouriotis, 2000). The effects of alcohol that are related to carbohydrates are decreasing glycolysis in a liver and brain, decreasing the tricarboxylic acid cycle, reducing the pentose phosphate pathway in the liver, increasing the pentose phosphate pathway in the brain, and

decreasing the metabolism of galactose, fructose, and sorbitol (Badawy, 1977b). I do not know if or how *Cda4* may contribute to the effect of ethanol on these pathways.

(vi) The effect of alcohol on drug transporting system

Monocarboxylate transporter 1 (Mct1) was down-regulated in Canton-S wild type flies after ethanol exposure (*Mct1*; $p < 0.05$, Table 1). The role of this transporter in a brain is to influence the metabolism of drugs such as alcohol by transporting the lactate and pyruvate across the biological membrane depending on the concentration of substrate in the extracellular and the intracellular environment (Vijay & Morris, 2014). When ethanol is oxidized by alcohol dehydrogenase to acetaldehyde, NAD is reduced to NADH (Badawy, 1977a), and the high concentration of NADH prevents the oxidation of lactate to pyruvate, which inhibits gluconeogenesis (Krebs, Freedland, Hems, & Stubbs, 1969). The possible effect of down-regulation in *Mctl* is to affect transporting the lactate and pyruvate across the biological membrane, which finally affects the metabolism of ethanol.

(vii) The effect of alcohol on Glutathione depletion

Glutathione S transferase E14 (*GstE14*) was down-regulated in Canton-S wild type flies after ethanol exposure (*GstE14*; $p < 0.01$, Table 1). It has been previously shown that acute ethanol administration depletes the level of glutathione secretion in older mice (Vogt & Richie, 2007). It is possible that in *Drosophila* the down-regulation of *GstE14* may impact glutathione levels, and hence ethanol detoxification.

4. Comparison among previous studies

Among the previous studies from Morozova et al., 2006, Urizar et al., 2007, and Kong et al., 2010, there were 29 genes were revealed to be differentially expressed in all three studies (Kong, Allouche, et al., 2010) (Figure 10).

Morozova, T. V., et al. (Figure 12) showed that expression of the genes encoding proteins for odor recognition such as proteins lush, *Obp19a, Pbprp1-5,* the odorant receptor *Or67d*, and the ubiquitous odorant receptor *Or83b* were down-regulated. Expressions of two olfactory proteins, *Obp99d* and *Pinocchio* were up-regulated. The expression of *Cyp6a2, Cyp6a13*, and glutathione-S-transferase D5, which encode biotransformation enzymes, were acutely up-regulated (Morozova et al., 2006).

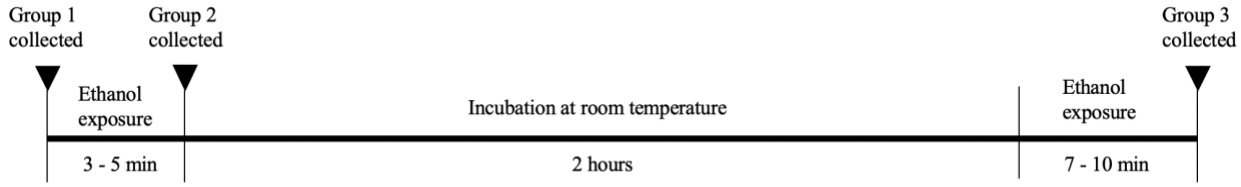


Figure 12. Treatment schedule for the Oligonucleotide microarray analysis from Morozova et al., 2006. Canton-S genetic background strain was frozen without ethanol exposure for the control group (group 1). The flies were exposed to ethanol vapor for 3 to 5 minutes until the flies were eluted from the inebriometer. The eluted flies were collected and frozen immediately (Group 2). 2 hours after the 1st ethanol exposure, the flies were exposed to ethanol vapor for 7 – 10 min until the flies were eluted from the inebriometer. Those flies were collected and frozen (group 3). Total RNA was extracted and analyzed by oligonucleotide microarrays. For data analysis of quantitative expression, post hoc Tukey tests were used at the $p < 0.05$ level (Morozova et al., 2006).

The study form Kong, E. C., et al. (Figure 13) showed that there was a regulation of expression for genes that function in olfaction, heat-shock responses, and immunity after ethanol exposure. For example, olfactory genes, including olfactory co-receptor *Or83b*, the *OBPs Lush* and *OS-E,* and the pheromone-binding proteins *Pbprp1, Pbprp3, Pbprp4*, and *Pbprp5* were down-regulated. Almost half of all *Drosophila Hsp* genes were significantly up-regulated

following ethanol exposure. The expression of immunity genes for the Toll (*cact, Myd88, Tl*), Imd (*imd, Rel*), and melanization (*Spn27A*) pathways were also highly expressed after ethanol sedation (Kong et al., 2010).

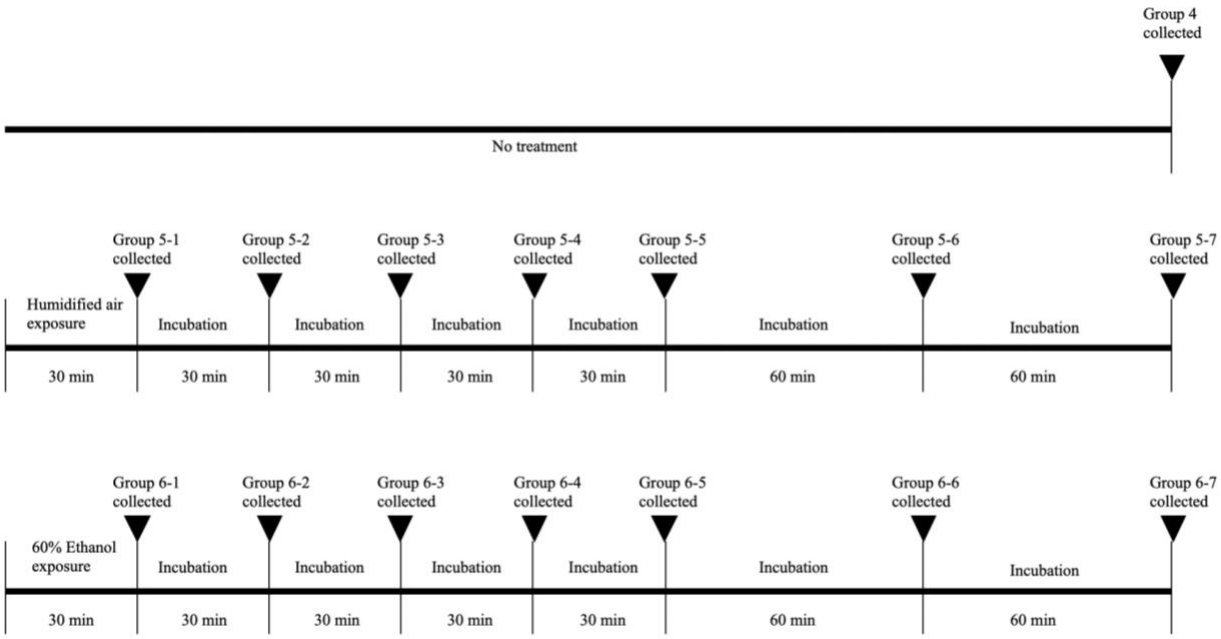


Figure 13. Treatment schedule for the Oligonucleotide microarray analysis from Kong et al., 2010. For the control group (group 4), flies of the Berlin genetic background strain were frozen without any treatment. For the experimental group, flies were exposed to either humidified air (group 5) or 60% ethanol vapor (group 6) for 30 minutes. The flies were frozen immediately, 0, 30, 60, 90, 120, 180, and 210 minutes after the ethanol vapor exposure. The RNA was extracted from their heads and analyzed by oligonucleotide microarrays. The quantitative gene expressions were analyzed by Limma package in the R statistical program. Differential expression between group 4 and group 6 at $p<0.05$ were clustered HOPACH algorithm (Kong, Allouche, et al., 2010).

The study from Nancy L. Urizar, et al., 2007 (Figure 14) showed 168 genes, encoding transcription factors, signaling proteins, RNA-binding proteins, and metabolic enzymes such as glutathione transferase and cytochrome P450, were differentially expressed (Nancy L. Urizar, 2007).

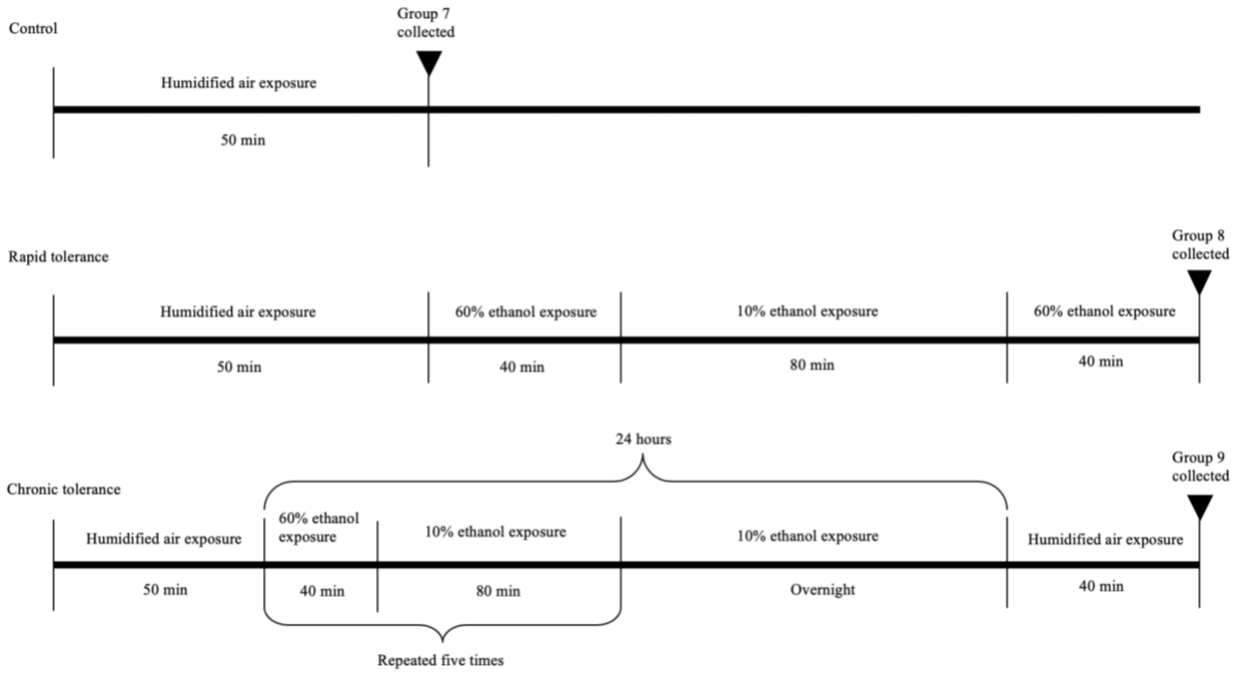


Figure 14. Treatment schedule for the Oligonucleotide microarray analysis from Urizar et al., 2007. The white-eyed Canton S (CS) line, *w*(CS10) genetic background strain were used in this study. For the control group, the flies were collected after 50 minutes of humidified air exposure (group 7). In this experiment, rapid and chronic tolerance were induced. For rapid tolerance (group 8), flies were exposed to 60% ethanol vapor after the 50 minutes of humidified air exposure. Then, they were exposed to 10% ethanol vapor for 80 minutes, followed by 40 minutes of 60% ethanol vapor, and then they were collected. For chronic tolerance (group 9), the flies were first exposed to humidified air for 50 minutes. After the exposure, they were exposed to 60% ethanol vapor followed by 80 minutes of 10% ethanol exposure, which was repeated five times. And then the flies were kept in vials containing food overnight for recovery. 24 hours after the first humidified air, the flies were exposed to humidified air for 40 minutes, and then they were collected (Nancy L. Urizar, 2007).

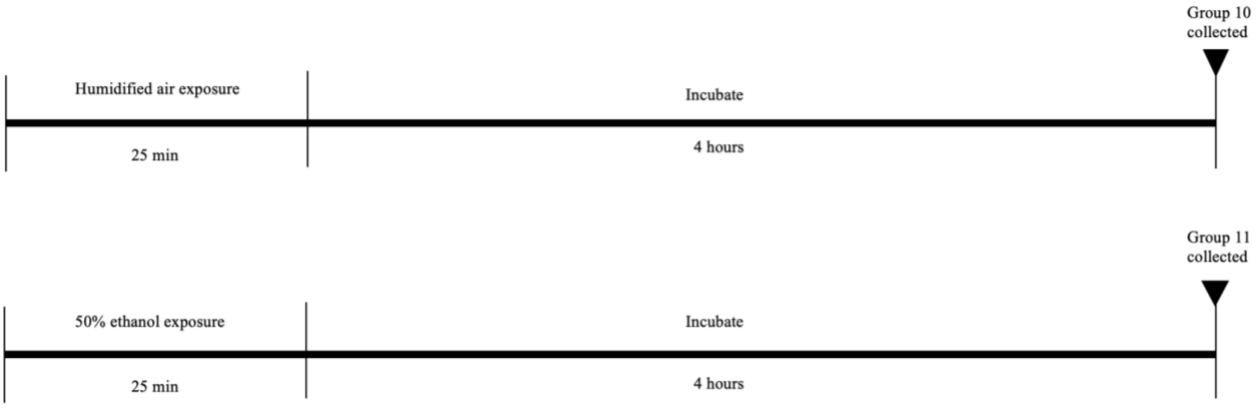


Figure 15. Treatment schedule for RNA-seq analysis in this study. Total RNA in each group were quantified by RNA-seq. Canton-S genetic background strain was exposed to either humidified air (group 10) or 50% ethanol vapor (group 11). For group 11, the flies were exposed to ethanol vapor until 90% of them in the assay were sedated, which approximately took 20 minutes, and for group 10, the flies were exposed to humidified air for the same amount of time as group 11 were exposed to ethanol vapor. After the exposures, both of the groups were incubated for 4 hours at room temperature, and then they were collected.

Altogether, there were more differences in the differential gene expression between studies than similarities. Several studies use different genetic backgrounds, ethanol exposure time, the concentration of ethanol vapor as shown in Figure 12 -15. Since gene expression is dynamic, depending on the age, incubation condition, and the protocol for ethanol exposure, there may be several small differences between the experiments that could significantly change the transcriptional profile of total RNA extracted from the heads of *Drosophila* used in these studies (Kong, Allouche, et al., 2010; Torres-Oliva, Schneider, Wiegleb, Kaufholz, & Posnien, 2018). Ultimately, examining the changes in the transcriptional responses to ethanol within a small subset of neurons critical to the sedative effect of ethanol may help identify the changes in gene expression that are critical for the formation of tolerance.

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