CAUSES OF NAIVE ETHANOL AVOIDANCE IN *DROSOPHILA MELANOGASTER*

by

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Lastly, a thank you to the Dean's Research Scholar for funding this endeavor.

ABSTRACT

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Background: *Alcohol dependence is a pressing public health concern, yet little is still known about its molecular causes. Although current studies have started to understand human addiction, Drosophila research is used as a tool to carry out more genetic and behavioral approaches that are crucial in learning about the addiction process.*

Objective: *The aim of this project was to understand the mechanisms of ethanol avoidance in Drosophila.*

Methods: *I applied quantitative ingestion assays to determine the amounts of food flies ate, with or without supplemented ethanol. I also used a choice assay, the FRAPPE, to determine whether naive flies exhibited preference for 15% ethanol. To interfere with neuronal function, I used the Gal4/UAS system, which allows for tissue specific manipulation of the activity of both neurons and genes.*

Results: *On average, Drosophila flies ate less sucrose when ethanol was added. One reason for this was that fewer flies initiated feeding. Upon silencing of gustatory neurons that perceive aversive tastes, flies showed less aversion to ethanol-containing food in the choice FRAPPE assay. As I increased the starvation time, almost all flies initiated feeding, but consumption amounts were still lowered when ethanol was supplemented. Additional feeding experiments where flies were only exposed to ethanol odor, but were unable to touch it, suggested that ethanol odor also suppresses food intake. I corroborated this with ethanol vapor exposures of defined intensity and duration: during the first minute of exposure, ethanol vapor stimulated food intake, but beyond that, it caused a suppression.*
Mutation in the ics gene affected ethanol-induced food suppression, but had no effect on the initial ethanol-induced stimulation of food intake.

Conclusion: *Drosophila flies show multimodal suppression of food intake by ethanol. Both the taste and smell of ethanol can reduce sucrose consumption. Interestingly, ethanol odor initially enhanced, but with continued exposure suppressed food intake. This suppression was abolished in ics mutants. This gene, whose human ortholog is linked to alcohol abuse disorders, is therefore critical for alcohol aversion, explaining how ics mutant flies show high, naïve preference for ethanol-containing food.*

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CHAPTER 1
An Introduction

From a shot of hard liquor during a celebration, to a glass of wine after a hard day's work, millions of people drink alcohol (ethanol) every day. Its effects help the shy to become social, the stressed to become relaxed, and the saddened to avoid their troubles. However, when people display as few as two of the 11 criteria for Alcohol Use Disorder listed in the Diagnostic and Statistical Manual of Mental Disorders, 5th edition, they qualify for this medical diagnosis, and the presence of six or more of these criteria merits a "severe" specifier (American Psychological Association, 2013). Society simply identifies these people as alcoholics. Alcoholism is seen in over 7% of the adult population and 2.8% of the youth population, creating a societal burden (NIAAA, 2016). People with an alcohol use disorder are at an increased risk for digestive diseases (especially in the liver), numerous cancers, cardiovascular disease, permanent neurological damage, and injuries and accidents. Medical emergency departments estimate 10-18% of injured patients are alcohol-related cases, yet worldwide, up to 45% of injured patients visiting emergency departments report consuming some amount of alcohol prior to their injuries (WHO, 2007). These percentages underscore alcoholism as a pressing public health concern. Insurance fees related to injuries and diseases, lost productivity and increased unemployment rates among alcoholics, and the amount of money spent on alcohol make alcoholism an economic burden. These problems and burdens have spurred global, national, and local organizations and labs to gather information to understand the effects of alcohol, in hopes of reducing the vast number of problems that alcohol can cause a person and a society.

Alcoholism, or alcohol dependence, begins with alcohol consumption (Grant, 2009).

Initially, acute low levels of alcohol activate the reward pathway in the brain through dopamine, leading to pleasurable effects. Many people enjoy the euphoric effects of alcohol, which may lead an individual to consume more alcohol in the future. Continued alcohol consumption forces the reward pathway to adapt. This adaptation involves up-regulating glutamate, the inhibitory molecule in the reward pathway, which increases the threshold at which the reward pathway can be activated. Therefore, not only are higher concentrations of alcohol needed to feel the previous pleasurable effects of drinking, but alcohol becomes needed on a regular basis to combat new feelings of stress, anxiety, or unhappiness that the newly inhibited reward system perceives (Koob & Volkow, 2010).

In addition to changes in the reward pathway, those who have a decreased ability to taste bitterness, or those whose bitter taste receptors are weak in signaling, tend to find that alcoholic beverages taste sweeter and that those individuals will go on to consume larger amounts of alcohol (Lanier et al, 2005). Likewise, if up to 95% of taste is derived from smell, then individuals who do not smell as well or have weaker smell receptors might find the aroma of alcohol less repugnant and might initially make an effort to consume more alcoholic drinks than an individual with a more sensitive sense of smell (Spence, 2015). This process suggests that there are numerous physiological pathways and reactions contributing to alcohol aversion. An alteration of any one of these systems may offer a clue as to why some individuals are initially more susceptible to alcohol and consequently more susceptible to alcohol addiction and dependence.

Genetic and environmental factors contribute to alcohol abuse and dependence. Not only does alcoholism run in families, but genetics contribute to over 50% of the risk to alcoholism.

Genes may even play a role in how effective certain treatments are in individual alcoholics (NIAAA, 2016). With such a strong genetic component, the study of alcohol abuse benefits from model systems that can easily be genetically manipulated and then further investigated with physiologic and molecular tools. *Drosophila melanogaster* fits these criteria due to the fact that it has been used since 1910 as an organism of study for heredity (Sturtevant, 1965). Historically, *Drosophila*, also known as the common vinegar fly, are a cost effective model system with a short life cycle and large progeny numbers. The *Drosophila* genome can be manipulated to create transgenic flies with the use of transposable element vectors to investigate a vast number of mutations (Spradling & Rubin, 1982). Many labs, including our own, use the Gal4-UAS system, a common method in flies, which can also be used in human cells, to manipulate flies in a tissue-specific manner. The Gal4 gene encodes a yeast transcription activator protein (Gal4) and its binding site, the Upsteam Activation Sequence (UAS), functions as the promoter to which Gal4 binds to and activates gene transcription for the gene of study (Brand & Perrimon, 1993). This method also allows us to study specific anatomical areas of interest within the fly system by expressing Gal4 in a tissue-specific manner, e.g. neurons, and it can further be applied to specific cell types, like olfactory sensory neurons. Additionally, these methods allow for mutants to be obtained and studied more easily.

Furthermore, *Drosophila melanogaster* have been used in behavioral analyses, some including feeding (Britton & Edgar, 1998), gustatory behavior (Chandrashekar, 2000), drug addiction habits (Rothenfluh & Heberlein, 2003), and learning and memory (Zong, 2006). In addition to the collected behavioral analyses, *Drosophila melanogaster* react to ethanol

similar to the way mammals do; low concentrations lead to hyperactivity and disinhibition, while high doses cause sedation (Ojelade & Rothenfluh, 2009). A single short high concentration exposure causes rapid tolerance, an acquired resistance, and a prolonged low concentration exposure causes chronic tolerance (Scholz et al., 2000). Therefore, ethanol pre-exposure leads to the development of ethanol preference (Devineni & Heberlein, 2009). Furthermore, flies will self- administer ethanol food, learn to like an ethanol-paired odor cue as it was previously associated with ethanol intoxication, and they will acquire an ethanol consumption preference when pre-exposed to ethanol vapors (Devineni & Heberlein, 2009) (Kaun et al., 2011) (Peru et al., 2014).

Although the vinegar fly and humans do not appear similar on the outside, they indeed share many fundamental biological mechanisms and pathways for development and survival (Jennings, 2011). A cross-genomic analysis of all known human disease genes and the *Drosophila melanogaster* genome illustrate that ~75% of those human disease genes share related sequences in the *Drosophila melanogaster* genome. Genetic homologues found in *Drosophila* have been linked to a vast number of human diseases, such as: cancer, cardiovascular disease, neurologic disorders, metabolic diseases, and genes required for visual, auditory, and immune systems (Bier, 2005).

In a genetic aspect, the development of human addiction can be inherited. Many of the gene variants identified in human addiction likely alter the specification and maintenance of neuronal connections in both mice and humans (Uhl et al., 2008). Hence, further connections can be initially studied in flies. For example, *Drosophila melanogaster* contain Ras suppressor 1 (RSU 1), encoded by the *icarus* gene, which regulates ethanol sensitivity and

preference. RSU1 works in an adult nervous system by regulating actin dynamics downstream of integrin and upstream of the Rac1 GTPase, both of which are already known to have a role in alcohol responses (Ojelade et al., 2015) (Rothenfluh et al., 2009). Similarly, humans contain RSU 1 polymorphisms that are associated with reward anticipation and alcohol consumption. Loss of *Drosophila* RSU1 leads to high naïve ethanol preference, while loss of RSU1 in the mushroom bodies, a brain section in *Drosophila* used in olfactory learning and ethanol reinforced odor preference, leads to the lack of naïve and acquired preference (Pitman et al., 2009) (Kaun et al., 2011) (Ojelade et al., 2015). *Drosophila* RSU 1 is required in distinct neurons to modulate naïve aversion to alcohol and acquired ethanol preference.

As previously stated, if ethanol naïve flies, which show a slight aversion to ethanol and ethanol containing food, are exposed over time, the exposure leads to alcohol preference (Peru et al., 2014) (Devineni & Heberlein, 2009). Flies are attracted to the smell of ethanol, which could partially mediate ethanol preference (Zhu et al., 2003) (Schneider et al., 2012). In contrast, *Drosophila melanogaster* are averse to the taste of ethanol (Devineni & Heberlein, 2009). Here, I wanted to further inquire if alcohol indeed suppresses acute food intake in flies and if so, to what extent taste and smell contribute to this suppression. A more nuanced understanding of the role of sensory inputs (taste, smell) and mechanisms for alcohol intake in *Drosophila melanogaster* may ultimately lead to transitional research that can decrease the public health burden and human cost associated with alcoholism.

CHAPTER 2
Experimental Procedures

Fly Stocks

Flies were grown and kept at 25˚C and 65% humidity on a standard mix of cornmeal, yeast, and molasses. Only male flies, about 2-10 days of age, were used for all behavioral assays to keep any analyzed behaviors as synchronized as possible since female flies show slightly different behavioral tendencies. The silenced bitter neuron flies, Gr66a>Kir were the product from a cross of a Gr66a-Gal4 line and an UAS-Kir1;Tub-Gal80^{ts} line. The flies were grown at 18˚C and then moved to 28˚C for two days to activate expression of UAS-Kir1. The olfactory mutants, Or83b>Kir, were created from Or83b-Gal4 and UAS-Kir1;Tub-Gal80^{ts} lines. The flies were grown at room temperature (22˚C) and moved to 28˚C for two days. The icarus mutant (*ics*) had two lines: the 1061 line which has a Gal4 P-element inserted into the *ics* gene and the X5 line which is an imprecise excision of the P-element. Both lines do not contain Ras suppressor 1 and both were grown and kept at room temperature. RNA interference (RNAi) is a tool used so that antisense RNA molecules can interfere with gene expression by inducing degradation of specific mRNA. The UAS-6113 line is an RNAi line in order to suppress *ics* (Ojelade et al., 2015). The lines of *ics*$^{10\text{-}61\text{-}Gal4}$/+>UAS-6113, Gr66a-Gal4>UAS-6113, MB-Gal4>UAS-6113, and 23E10_Gal4>UAS-6113 were created by crossing neuron-specific Gal4 lines to UAS-6113. These crosses were grown at room temperature or 25°C and kept at 25° for 2 days prior to testing.

Ethanol consumption preference (termed FRAPPE: fluorometric reading assay of preference primed by ethanol)

In ethanol preference experiments flies were given a choice for 30 minutes of either 340 mM sucrose solution or a 340 mM sucrose solution with ethanol, caffeine, or denatonium. Flies were food deprived for 6 hours in a vial containing 400 uL of water absorbed in the top cotton stopper to increase consumption. Fluorescent dyes Rhodamine B (pink) 0.1% stock, 0.005% working solution, and Fluorescein (yellow) 0.3% stock, 0.003% working solution were used to label the two sucrose solutions. One plate contained the sucrose only solution colored with Rhodamine B and the experimental solution with sucrose contained fluorescein. Opposite color pairing was used in a second plate to counter balance any color biased potential the flies may have experiences during the preference assay (Peru et al., 2014). An orange plate containing a 1:1 ratio of the two fluorescent dyes was tested alongside each experiment to eliminate varying intensity ratios that occur between the two dyes. After the 30 minute feeding period, the plates were placed in a -20˚C freezer overnight. The next morning, flies were placed in 15 mL Falcon tubes, placed on dry ice for 5 minutes, and vortexed to separate the autofluorescing heads from the thorax and abdomen containing the choice dyes. Eight headless flies each were then placed into four 1.5 uL centrifuge tubes and 80 uL of water added to each of the four tubes. Flies were then ground and centrifuged for eight minutes. The 60 uL of the supernatant was placed in a 96 well flat bottom tissue culture plate and the plate was then placed in the Fluroskan. Using Ascent Software v2.6, fluorescence data was collected in a Fluoroskan Ascent FL2.4 plate reader at excitation/emission wavelengths of pink 542/591 nm and of yellow 485/527 nm. A preference index (PI) for each

individual fly was calculated (nl consumed of sucrose/15% solution—nl consumed of sucrose)/(nl consumed of sucrose/15% solution + nl consumed of sucrose), ranging from +1 =total preference, to −1= total avoidance (Peru, 2014).

Ethanol feeding assay

Desired flies were collected and placed in food agar vials overnight to recover from CO_2. Clean, empty vials were set up with 400 uL water pipetted on the inside of the cotton topper to keep flies hydrated during starvation period. Flies were transferred into the empty vials for 6, 8, or 12 hours. New empty tubes were set up with 3.5 x 1.75 cm filter paper strips equidistance from top and bottom with 350 uL of desired solution pipetted onto the strip. Desired solutions contain 0.3% blue dye for identifying which flies ate later in analysis. After the starvation period, flies were transferred into blue food vials and allowed to eat for 4 minutes. The flies were then transferred back to their original starvation vials and placed in -20˚C freezer overnight. The next morning, flies were placed onto a light microscope pad and the total fly number and number of flies that ate (flies with blue bellies) were recorded. Five flies which ate were added to a 1.5 uL Eppendorf tube. Fifty uL of water were pipetted into each Eppendorf tube. Flies were ground and centrifuged for 5 minutes. Two uL from each tube was analyzed using the NanoDrop computer program specifically recording values at 630 nm and 700 nm for the blue dye in the food. An absorbance conversion factor was used: 26.8 = 100nL per fly.

Olfactory trap assay

Trap assays were conducted in a Petri dish (Fisher 100 mm x 15 mm). Traps were constructed from 1.5 mL centrifuge tube (with a flat cap) and two 20-200 uL pipette tips. The

end of the centrifuge tube was severed with a razor blade approximately 2.5 mm from its narrow end. Each pipette tip was severed 0.8 cm from its narrow end. One of the two tips was severed additionally to separate the rigged and smooth portions. The twice cut tip was placed inside the centrifuge tube (small end inside) and the other tip placed over the exposed first tip (small end facing outside; Woodward, 1989). Ten flies were anesthetized with CO_2 and placed into a petri dish containing a trap. The number of trapped flies was periodically recorded while ending at a maximum of 30 hours or until all flies were in the trap and/or dead. Petri dishes/traps were kept at room temperature (22˚C).

Booze-O-Mat Feeding Assay

The Booze-o-mat system tubes were modified by making three small holes in the side of the tubes to fit a 1000 uL pipette tip end. An 8 x 4 inch piece of paper was placed in the tubes before flies were added and put in the Booze-o-mat. At different time points, the ethanol exposed flies were given 0.3% blue dye solution of 400 mM sucrose evenly dispensed through the three small holes. Time points used were 0-1 minute, during which flies show olfactory startle-mediated hyperlcocomotion, 2-3 minutes, when flies have habituated to the smell are more quiescent, and 5-6 minutes when ethanol vapor in the brain of the *Drosophila* fly causes hyperactivity (Rothenfluh & Heberlein, 2002). Different ethanol/air flow rates were also used to study the effect of ethanol of feeding. Flies were allowed to eat for 1 minute then the tube was taken out and covered with tape and put in a -20˚C freezer overnight. The next morning, flies were placed onto a light microscope pad and the total fly number and number of flies that ate (flies with blue bellies) were recorded. Five flies that ate were added to a 1.5 uL Eppendorf tube. Fifty uL of water was pipetted into each Eppendorf

tube. Flies were ground and centrifuged for 5 minutes. Two uL from each tube was analyzed using the NanoDrop computer program specifically recording values at 630 nm and 700 nm for the blue dye in the food. An absorbance conversion factor was used: 26.8 = 100nL per fly.

CHAPTER 3
Results

Consumption

During an ethanol feeding assay, *Drosophila* flies consumed significantly less sucrose when increasing amounts of ethanol were added to the food source (Fig 1A left). This was accompanied by a decrease in feeding initiation with increasing ethanol/sucrose ratios (Fig 1A right). To examine whether this decrease in volumes consumed was wholly explained by a decrease in initiation, I performed another ethanol feeding assay. In this assay flies were deprived for increasing amounts of time, such that almost all of them would initiate feeding once presented with the alcohol/sucrose solution. In addition, in this assay I calculated the amounts of sucrose eaten normalized to the number of flies that did eat, i.e. flies that showed no ingestion were excluded from the ingested average volume. These data showed that even under conditions where almost all flies ate, ethanol still reduced intake volume (Fig 1B,C). This suggested that a decreased consumption of ethanol food was not solely accounted for by a decreased in initiation.

To investigate whether ethanol odor contributed to the decreased consumption, I performed another assay, during which sucrose and ethanol were provided on two separate strips, such that flies would not be in physical contact with ethanol when consuming sucrose. Compared to the ethanol/sucrose mixture, simultaneous, but separate presentations of ethanol and sucrose led to a reduction in initiation, but still significantly reduced the volume ingested (per fly that ate, Fig A). This suggested the marked reduction in initiation required physical touch of the ethanol, possibly mediated by taste neurons.

Taste

Drosophila taste modalities include sweet, bitter, water (osmoles), and carbonation. Each of these modalities is sensed by a defined set of gustatory neurons, but within the group of bitter neurons, each bitter gustatory sensory neuron can express multiple receptors for various bitter compounds, such as caffeine or denatonium. It has previously been shown that vinegar flies do not like the taste of ethanol or ethanol-sucrose foods (Devineni & Heberlein, 2009). To determine whether this is mediated by bitter neurons, I electrically silenced (Gr66a-Gal4 expressing) bitter neurons by overexpressing an inwardly rectifying potassium channel (UAS-Kir1). These Gr66a>Kir transgenic flies were used in an ethanol consumption preference or FRAPPE assay (Fig. 2B). As expected, Gr66a>Kir flies disliked caffeine less as the preference index (PI) for a caffeine containing sucrose solution was no longer negative. The same flies also showed a significantly less negative PI for a 15% ethanol containing sucrose solution, indicating that ethanol was indeed perceived by canonical bitter gustatory neurons of flies.

Smell

The two strip feeding assay (Fig. 2A) also included an assay where a physical mesh barrier was placed between the ethanol and sucrose strips. This would prevent the flies from ever touching the ethanol. Still, the ethanol odor significantly reduced the volume of sucrose ingested, suggesting that ethanol odor itself would reduce sucrose consumption. This was somewhat surprising in light of reports that flies are attracted to the smell of ethanol (Zhu et al., 2003). To investigate this further, I first performed an olfactory trap assay with wild type *w Berlin* flies to establish if flies indeed were attracted to the smell of ethanol. I observed that

w Berlin flies were attracted to a trap with ethanol, even up to a concentration of 35% (Fig. 3). The olfactory trap assay did illustrate that flies liked the smell of ethanol but did provide information about how the smell of ethanol effected consumption. To determine how the smell of ethanol affects sucrose consumption, I designed an experiment that allowed me to expose flies to defined doses and times of ethanol smell. This experiment was based on the booze-o-mat, where flies are exposed in tubes to predetermined ratios of vaporized ethanol and water streams (Wolf et al., 2002). I found that high doses of ethanol vapor actually increased sucrose consumption during the first minute of exposure (Fig. 4A). However, when feeding flies after 2 minutes of exposure, when they have habituated to the startle-inducing smell of ethanol (Rothenfluh & Heberlein, 2003), they showed ethanol-induced suppression of food intake (Fig. 4B). After 2 minutes of ethanol exposure flies, habituated flies show less locomotion and are relatively quiescent (Wolf et al., 2002). To investigate whether this hypoactivity was the cause for the reduction in sucrose consumption, I next assayed flies after 5 minutes of ethanol exposure. At this time of exposure, flies show ethanol-induced hyperlocomotion, which is not smell-mediated, but is caused by the direct pharmacodynamic action of ethanol on the brain (Wolf et al., 2002). Again, ethanol suppressed food intake, arguing that locomotion activity and the effect of ethanol smell on sucrose consumption were not correlated. These results showed that the effect of ethanol smell on sucrose consumption was biphasic, first activating consumption then over time suppressing consumption.

To further test if smell had an effect on consumption, I used Or83b-Gal4 > Kir flies to silence the majority of olfactory neurons. The Or83b gene encodes for a broadly expressed odorant co-receptor required for *Drosophila* olfaction and the Kir silences the olfactory neurons

(Larsson et al., 2004). The smell mutant consumed less during a consumption-enhancing exposure to ethanol vapors, suggesting that the smell of ethanol did increase consumption of a food source in flies (Fig. 5).

Icarus

icarus mutant flies lack the Rsu1 protein, and this includes the two alleles ics^{X5} and ics^{1061}, latter of which expresses Gal4 driven by the endogenous *ics* promoter (Ojelade et al., 2015). In the ethanol feeding assay, *ics* mutants did not show a decrease in consumption when ethanol was added to sucrose, consistent with their absent naïve alcohol avoidance (Ojelade et al., 2015). This phenotype was rescued by the reintroduction of a *UAS-Rsu1* transgene driven by ics^{1061} (Fig. 6). Unlike control flies, *ics* mutants displayed no avoidance to ethanol containing solutions in the FRAPPE (Fig. 7).

This lack of ethanol avoidance could be caused by *ics* mutants' blindness to the bitter taste of ethanol, their refraction to ethanol odor-induced consumption suppression, or a combination thereof. When *ics* mutant flies were placed in the olfactory trap, they were attracted to increasing ethanol concentrations more strongly than control flies, such that not only did more flies go into the trap, but the flies accumulated in the trap in a shorter time interval (Fig. 8). This suggests that *ics* mutants do not have a general inability to perceive ethanol odors. Booze-o-mat feedings were performed in order to test whether ics mutants showed a similar biphasic response to ethanol odor as wild type, i.e. initial enhancement, followed by suppression of consumption. *ics* mutant flies exhibited increased consumption when exposed to ethanol during the 0-1 minute feeding interval; however, they did not exhibit the normal inhibitory phase of smell, seen in *w Berlin* flies, during which

consumption is dramatically decreased (Fig. 9).

Neurons Involved

In order to determine where the *ics* gene and its products were required for normal ethanol aversion, *ics-RNAi (UAS-6113)* lines knocking down *ics* gene product (RSU1) were crossed to different neuroanatomical Gal4 drivers. The *ics1061>6113* line were flies that had an *ics* knockdown in all cells that expressed *ics* in *Drosophila*. This line showed the same FRAPPE results as the *ics* mutant flies did, where the flies were less averse to ethanol containing solutions (Fig. 10). The second knockdown was in the bitter neurons, *Gr66a>6113,* and those flies illustrated the same FRAPPE results as the previous line, *ics1061>6113* (Fig. 10). The third knockdown was in the mushroom bodies, *MB>6113,* which led to ethanol avoidance (a negative PI) in the FRAPPE much like in control flies (Fig. 10). The last knockdown occurred in the fan shaped bodies, *23E10>6113,* and those flies displayed a less averse FRAPPE result when given a choice between ethanol-sucrose and sucrose (Fig. 10).

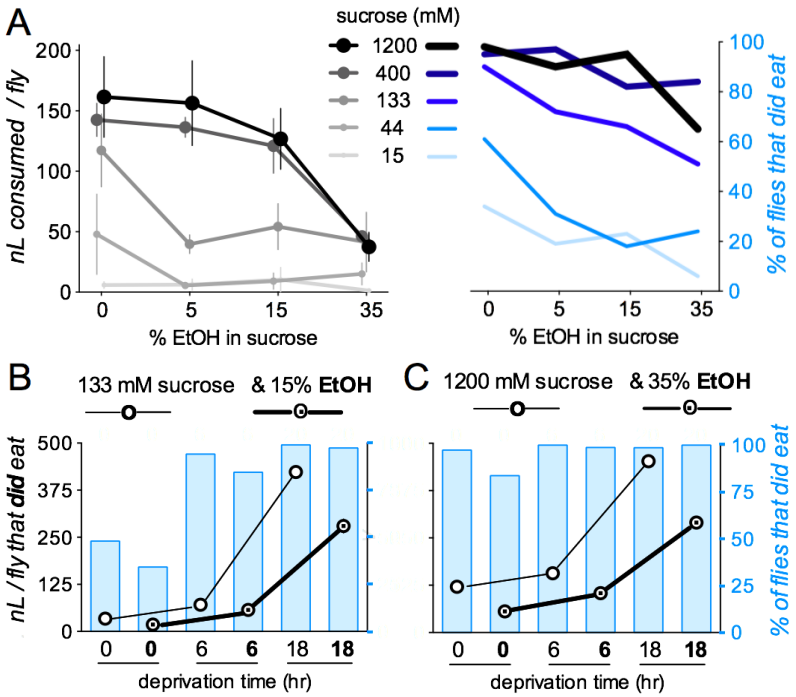

Figure 1. Ethanol-sucrose consumption as a function of ethanol concentration and deprivation time: (A) Both the amount eaten (left) and the initiation (right) decreased with increasing percentages of ethanol added in differing concentrations of sucrose in the ethanol feeding assay with *w Berlin* flies (B) ethanol containing sucrose decreased consumption for all deprivation times and decreased initiation when flies (*w Berlin*) were deprived of food for less than 18 hours (C) ethanol containing sucrose decreased consumption for all deprivation times in *w Berlin*, yet initiation was not decreased when the higher sucrose concentration of 1200 mM was used.

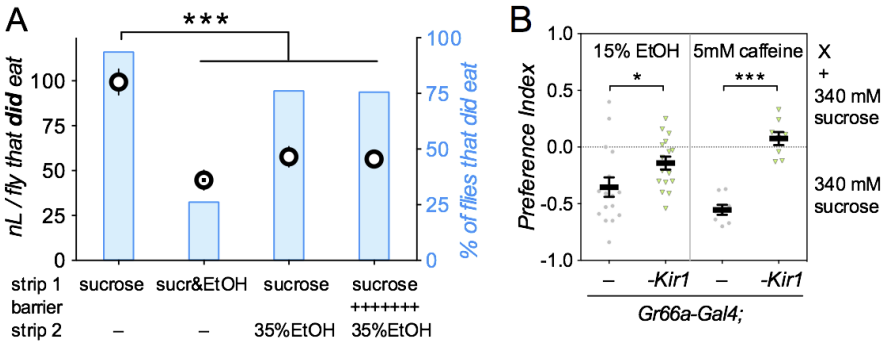

Figure 2. Sensory modalities in decreased ethanol-sucrose consumption: (A) Ethanol mixed with sucrose decreased consumption and initiation drastically, suggesting an aversive taste, while ethanol and sucrose on different strips decreased initiation moderately, suggesting an aversive smell in the ethanol feeding assay with WB. (B) Flies with silenced bitter taste neurons (Gr66a-Gal4>Kir1) demonstrated taste blindness (PI close to 0) to the control bitter compound of caffeine and were less aversive (less negative PI) to the tested ethanol when compared with the control (*Gr66a-Gal4*) in the FRAPPE. Significant is a $p<0.05$.

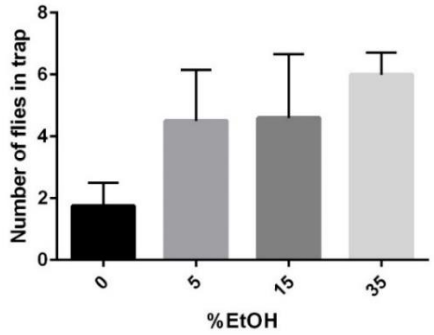

Figure 3. WB in an olfactory trap with ethanol as attractant: The flies were attracted to the smell of increasing ethanol concentrations as shown by the increased number of flies in the trap after 24 hours.

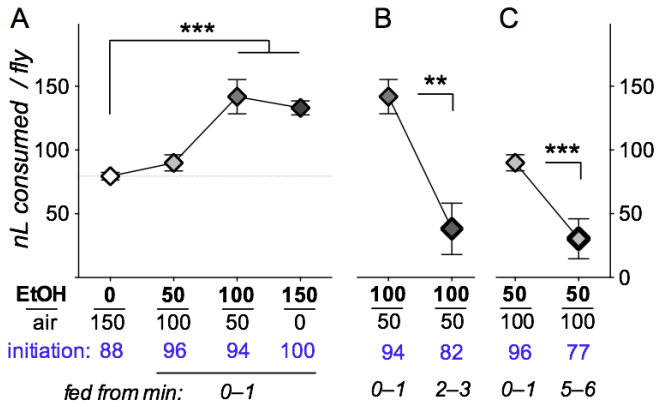

Figure 4. Effects of ethanol vapors on consumption: (A) During the *Drosophila* smell interval (0-1 minute), increasing parts of ethanol to air increased the amount of sucrose consumed while keeping initiation high in WB flies in the Booze-o-mat. (B and C) Feeding WB flies during habituation of smell (2-3 minutes) and once the ethanol vapors have reached the brain (5-6 minutes) led to a decreased consumption but also a decreased initiation. Significance is a $p<0.05$.

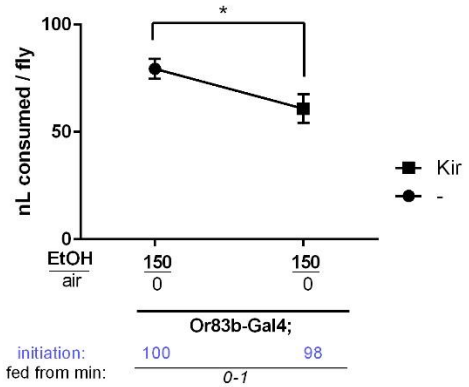

Figure 5. Smell mutant's consumption phenotype when exposed to ethanol: When exposed to 150 parts of ethanol to 0 parts of air, the smell mutant Or83b>Kir experienced decreased consumption but not decreased initiation during the smell interval feeding of 0-1 minute when compared to the control.

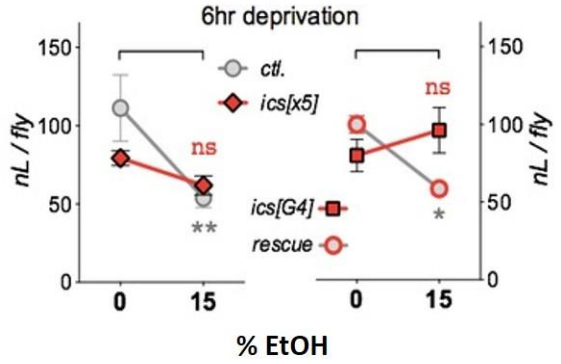

Figure 6. *Ics* mutant phenotype in ethanol feeding assay: *Ics* mutants do not experience a decreased consumption (nL consumed per fly) when ethanol is added to sucrose; this phenotype can be genetically rescued (right).

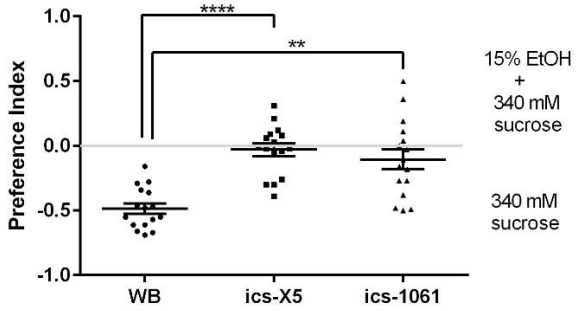

Figure 7. *Ics* mutants do not show naive aversion to ethanol: When compared to the control (WB), both *ics* mutants, X5 and 1061, demonstrate no naive aversion to ethanol during a FRAPPE assay as the PI was not different from 0. Significance is a $p<0.05$.

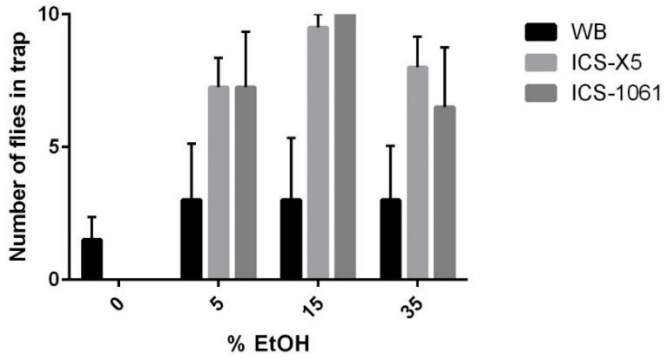

Figure 8. *Ics* mutants in olfactory trap with ethanol as attractant: Data from the trap was collected after 18 hours to show that *ics* mutants were attracted to increasing ethanol concentrations more quickly and in greater number than the control flies (WB).

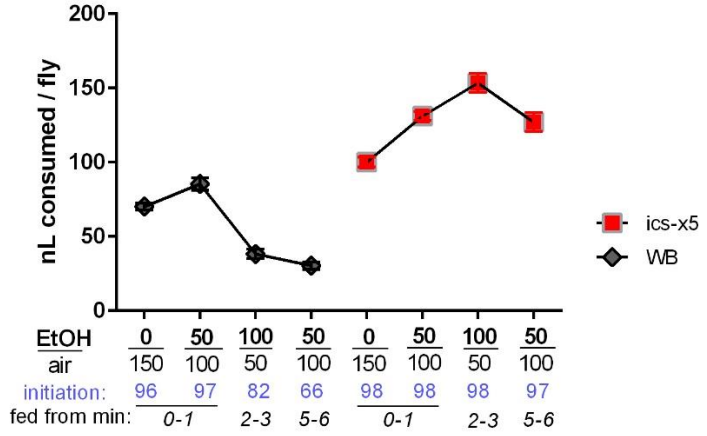

Figure 9. *Ics* mutants lack an inhibitory smell phase: At the 2-3 or 5-6 minute intervals during exposure to ethanol, WB flies showed a decrease in consumption that goes below the 0/150 (no ethanol) baseline. In addition, they demonstrated a decrease in initiation at that those time points as well. However, during the same intervals, *ics* mutants did not demonstrate the decreased consumption below that of the baseline nor the decreased initiation.

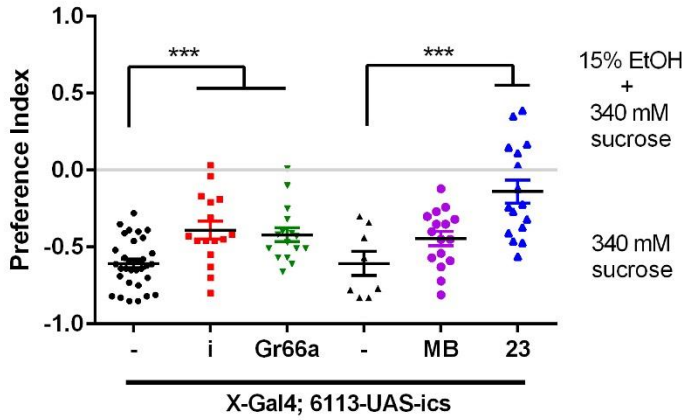

Figure 10. Naive increased ethanol preference mutants requires *ics* in the the fan-shaped body: The *i>6113* line, which knocked down *ics* in all *ics* containing cells, showed an increased preference (less negative PI) when given the choice of ethanol containing sucrose and sucrose. The Gr66a>6113 line, which knocked down *ics* in bitter neurons, showed an increased preference as well. The MB>6113 line, which knocked down *ics* in the mushroom bodies, showed no significant change from the control. The 23>6113 line, which knocked down *ics* in the fan-shaped body, showed an increased preference for ethanol containing sucrose. Significant is a $p<0.05$.

CHAPTER 4
Conclusions and Recommendations

Alcohol dependence is a severe health concern in the United States. Almost half of hospital emergency room visits due to injury are linked with alcohol drinking (WHO, 2007). Alcoholics are not only addicted to the consumption of alcohol, but are also at risk for passing on the genetics of that addiction to offspring. Humans consider alcohol to be bitter and they usually experience an awful taste during its consumption, yet social drinking, binge drinking, and alcohol addiction continues. If alcohol addiction begins with consumption, then the study of how initial consumption is regulated should be a priority. Due to the genetic component of alcohol dependence, some individuals are more susceptible to alcohol and its later addicting effects. If we work under the assumption that taste, smell, and the reward pathway are all part of an initial reaction to alcohol, then studying these three modalities in relation to alcohol's effects on consumption may give some insight into how an individual who is predisposed to an alcohol use disorder turns their alcohol consumption into alcohol dependence.

Initially, it was observed that ethanol decreased the amount of consumption in *Drosophila* flies, not by decreasing initiation but by decreasing the amount of food consumed. At first, both states of ethanol in sucrose and ethanol separated from sucrose by a mesh barrier showed a decrease in initiation and consumption. This result led us to hypothesize that taste and smell negatively impact consumption in flies. Previous literature results have shown that when ethanol is added to sucrose solutions, the proboscis extension reflex decreases, as the fly is avoiding ethanol-containing solutions (Devineni & Heberlein, 2009). It can then be hypothesized that flies, in general, do not like the taste of ethanol;

however, this does not identify which of the taste perceptions in flies registers ethanol as aversive. Since humans interpret ethanol as bitter and most aversive compounds in flies are known to go through the bitter perception pathway, then it was logical to test flies with silenced bitter neurons against solutions that contained a bitter compound and then an ethanol compound. The same flies which were no longer averse to caffeine, a bitter compound, were also no longer averse to ethanol, indicating that at least parts of ethanol perception indeed do go through the *Drosophila* bitter pathway, which is known to create such aversions to many solutions in flies. This finding confirmed past research, indicating that *Drosophila* flies did not like the taste of ethanol. An important next step to advance our understandings of these pathways will be to test the silences bitter neuron flies in a new preference assay to determine if the FRAPPE results of less avoidance were due to initiation or consumption.

Similarly, previous research examining taste information indicated that vinegar flies enjoyed the smell of ethanol, at least in small doses, most likely due to an evolutionary journey in order to seek out and find rotting fruit (Zhu et al., 2003). In addition, preponderance of evidence has indicated that *Drosophila* flies are attracted to ethanol when placed in an olfactory trap, which we replicated. To extend this finding, we examined how the smell of ethanol affected sucrose consumption. Contrary to our initial hypothesis, we found that smell increased the amount of food flies ate. This finding makes intuitive sense because if the smell of food is appealing, then most creatures would enjoy eating more of that particular food. This finding is also consistent with the account that up to 95% of taste is derived from smell (Spence, 2015). However, *Drosophila's* locomotion-activating effects of smell habituates after a little over a minute of exposure, so it remains unclear how smell

affected feeding after a minute of being surrounded by an odor. Habituation of smell in vinegar flies is considered to be in the 2-3 minute period, and here, ethanol vapors quickly reduced consumption through initiation and amount consumed. To our surprise, we saw biphasic effects of smell. Initially, the ethanol smell increased the amount of food consumed, but later that same smell had an inhibitory effect on attempting to consume food and on the amount of consumed food. This was further supported by the observation that a fly without smell, a smell mutant, did not eat as much in the first minute of an ethanol aroma as a normal fly would. Future directions for this research will be to test the smell mutant in the 2 minute and 5 minute time points in the Booze-o-mat feeding assay to evaluate the evidence of the inhibitory phase for this hypothesis.

Furthermore, unlike the ethanol aversive mechanisms carried out by wild type flies, the *ics* mutant flies were blind to the aversive taste of ethanol but strongly preferred the ethanol in a long-term 2-bottle choice paradigm (Ojelade et al., 2015). However, even though the *ics* mutants demonstrated increased consumption with ethanol vapors, the smell-induced inhibitory phase was not seen, leading the mutants to increase sucrose consumption throughout the habituation and brain time periods of ethanol exposure. After contemplating the unique *ics* mutant phenotype, RNAi was used to knockdown the *ics* gene products in order to determine which part of the ethanol sensory pathway was becoming distorted with the absence of the *ics* gene products (RSU1). Decreased ethanol aversion was seen in the *ics* knockdown in the fan-shaped body but not the mushroom bodies, indicating that the fan-shaped body is needed for naive ethanol avoidance in flies but the mushroom bodies are not. This data is strengthened by previous research which illustrated that normal naïve avoidance

was still present when RSU1 was knocked down in the mushroom bodies (Ojelade et al., 2015). The *ics* mutants have a unique combination of taste blindness and a strong activating smell phase which makes them initially more prone to higher ethanol-sucrose consumption. In addition, *ics* mutants most likely have an atypical brain mechanism, partly located in the fan-shaped body, which eradicates their inhibitory smell phase and allows for continued ethanol-sucrose consumption even after the intoxicating effects of ethanol have reached signals in the brain. Future directions would be to rescue *ics* in the fan-shaped body and carry out FRAPPE and Booze-o-mat feeding assays to determine if the *ics* mutant phenotype is then rescued as well. Most importantly, once the mechanisms of the *ics* mutants' ethanol tolerance come to light, further transitional research could be carried out as many alcohol dependent humans are linked to disfigured RSU1 products.

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VITAE

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