2020

MEF2-BOUND GENES MAY INFLUENCE ETHANOL SEDATION IN DROSOPHILA MELANOGASTER

Katlyn M. Myers
Virginia Commonwealth University

MEF2-BOUND GENES MAY INFLUENCE ETHANOL SEDATION IN *DROSOPHILA MELANOGASTER*

A thesis submitted in partial fulfilment of the requirements for the degree of Master of Science at Virginia Commonwealth University.

By
Katlyn Marie Myers
Bachelor of Science, Longwood University-Farmville, 2018

Advisor: Mike Grotewiel, PhD
Associate Dean for Graduate Education
Associate Professor, Department of Human & Molecular Genetics

Virginia Commonwealth University
Richmond, VA
July, 2020

Statement of Contributions

My thesis project was to explore Mef2-bound genes in ethanol sedation (chapter 3). Dr. Silviu Bacanu (Virginia Institute for Psychiatric and Behavioral Genetics, Department of Psychiatry, Virginia Commonwealth University) performed a GSCAN analysis on human orthologous genes of the 342 *Drosophila* genes that are known to be bound by Mef2. Dr. Grotewiel converted the results of Dr. Bacanu's GSCAN results back into the *Drosophila* genes. Dr. Grotewiel and I designed follow-up experiments on Dr. Bacanu's results (chapter 3). Other than Dr. Bacanu's and Dr. Grotewiel's analyses noted here, I performed all of the experiments and generated the other data shown in this thesis. Forward thinking future experiments (chapter 4) were also designed by myself along with assistance from Dr. Grotewiel.

Acknowledgements

My seemingly short time at VCU has been the longest, best, hardest, most rewarding experience of my life. The things I have learned have truly helped me become the scientist I have always wanted to be.

I would like to thank Dr. Grotewiel for his guidance throughout my graduate training and especially his patience. Words cannot describe how much I appreciate your patience throughout my time in the lab. I could not have picked a better academic advisor to help me through this process and always keeping me excited about science. Thank you for always having my best interest at heart.

I would also like to thank my committee members, Dr. Jill Bettinger, Dr. Andy Anderson and Dr. Laura Mathies for the support, and guidance through my graduate training.

Additionally, I would like to thank Dr. Mathies for use of her PCR equipment and reagents to aid with the transposon data presented in chapter 3. I also want to thank her for also going back to the basics of PCR.

I would also like to thank the VCU microscope core for training and image analysis assistance with data presented in chapter 3. I want to thank everyone in the Molecular Biology and Genetics program, along with Human and molecular genetics department.

I also want to mention past and current lab members who have helped me on this journey. Brandon shell for all our great lab memories (eating, Pokémon go, complaining, etc.) along with past members Dr. Becky Schmitt and Dr. Kristen Lee. Thank you, Kristen, for all your help and assistance with my fly brain troubleshooting and analysis even though you are on the other side of the country, I truly appreciate it!!!!!!!!

I also want to thank my best friends in the entire world, Lauren and Juli (yes Juli, I love you more). You guys make my life so much better and brighter and I could not have gotten through this process without you guys to lean on. I love you both more then you know.

I want to thank my mom and dad for their constant support (mentally and financially) I love you both so much and can never repay the impact you have had on my life. Thank you both for helping me learn to manage my time wisely, writing papers (thanks daddy!!), and making good life decisions. Kristen and Morgan, my not so baby sisters, for always hanging out with me and supporting me even when we fought. I love you all and your support has meant the world to me.

Finally, I would like to thank Longwood University's faculty, staff, and student body. When I stepped onto that campus six years ago, I never dreamed of going to graduate school. Without the opportunities in research and the people I met there (Dr. Shanle, Dr. Yeagley, Dr. Andy Anderson, my amazing study buddy and my other half, Mackenzie Tabler), I do not think I would be defending a master's thesis today. Thank you, and go wood!

Table of Contents

List of Tables

,

List of Figures

List of Abbreviations:

ANOVA……Analysis of Variance

AUD……Alcohol Use disorder

AUDIT……..Alcohol use disorder identification test

AUDIT-C……Alcohol use disorder identification test for consumption

AUDIT-P……Alcohol use disorder identification test for dependence

AUTS2…… Activator of Transcription and Developmental Regulator

BMC……..Bonferroni Multiple Comparisons

DSM…….Diagnostic and Statistical Manual of Mental Disorders

dsRNA……..double-stranded RNA

elav…….embryonic lethal abnormal vision

EBV……..Epstein-Barr virus

EGFR……Epidermal growth factor

Fwd……Forward

GDP……*Drosophila* Gene Disruption Project

GS……..GeneSwitch

GSCAN……..GWAS and Sequencing Consortium of Alcohol and Nicotine

GWAS……..Genome Wide Association Study

HAP1……High alcohol preference

Hr38…….Hormone receptor-like in 38

Hsp……Heat Shock Protein

Hsp26……..Heat Shock Protein 26

InR……..Insulin-like receptor

LAP1……Low Alcohol preference

LOF……..Loss of Function

MADS……MCM1, agamous, deficiens, serum response factor

Mef2……*Myocyte Enhancer Factor 2*

MEF2A, B, C, and *D*……*Myocyte Enhancer Factor 2A, B, C,* and *D*

PAU……..Problematic alcohol use

Polyhomeotic……..*ph*

Polyhomeotic distal……...*ph-d*

Polyhomeotic proximal……*ph-p*

Rev……..Reverse

RISC……RNA silencing complex

rTA……tetracycline Repressor Mutant

RNAi……RNAi interference

RSU1……...*Ras Suppressor Protein 1*

RU486……..Mifepristone

SNP(s)……Single Nucleotide Polymorphism(s)

SRE……..Self-Rating of the Effect of Alcohol

ST50……Sedation Time 50

Tay……*tay bridge*

TIR……..inverted repeats

TetO……..Tetracycline Operator

TetR…….Tetracycline repressor

UAS……Upstream activating sequence

XRCC5……X-ray repair cross-complementing protein

Abstract

MEF2- BOUND GENES MAY INFLUENCE ETHANOL SEDATION IN *DROSOPHILA MELANOGASTER*

Katlyn Marie Myers

A thesis submitted in partial fulfillment of the requirements for degree of Master of Science at Virginia Commonwealth University.

Virginia Commonwealth University
Advisor: Mike Grotewiel, PhD
Associate Dean for Graduate Education
Associate Professor, Human & Molecular Genetics

Alcohol Use Disorder (AUD) is a global health issue that affects millions of people every year. This disorder has serious negative mental and physical consequences. Currently, treatment options for this disorder are largely limited to psychological therapy, with very few medications available to treat it. Being able to identify the environmental and genetic components that influence AUD can help improve diagnosis and treatment options. Previous studies in humans have shown a link between initial sensitivity and risk for alcohol abuse. Our laboratory uses *Drosophila melanogaster* as a model to study the genetic and environmental components of alcohol-related behaviors. Previous lab members found that the transcription factor Mef2*,* the *Drosophila* ortholog to the *MEF2B* gene in humans*,* has a significant role in ethanol sedation in flies. We consequently predicted that genes regulated or bound by Mef2 in flies may also have a significant impact on ethanol sedation. Initial analysis of several candidate genes revealed that transposon insertions and neuronal RNA interference (RNAi) against *spinster* (*spin*) altered ethanol sedation. These genetic analyses, in combination with published data, support the hypothesis that *spin* influences ethanol sedation and does so by functioning downstream of *Mef2.*

Chapter 1-introduction

A. Alcohol Abuse and Alcohol Use Disorder

1. Alcohol Use Disorder

In 2016, more than half of the world's population (3.1 billion people) said that they currently drink alcohol (Hammer et al., 2018). Since then, the number of people who consume alcohol has only increased (Hammer et al., 2018). Consuming alcohol can often lead to alcohol abuse and alcohol use disorder (AUD). Alcohol abuse is a pattern of drinking too much alcohol too often and frequently interferes with a person's daily life (Anon, 2020). AUD is more complex and is defined as a problematic pattern of alcohol use that has many clinically significant impairments or distress outlined in the Diagnostic and Statistical Manual of Mental disorders (DSM) (Kranzler and Soyka, 2018). An estimated 15 million people in the United States 18 years old and older had some form of AUD in 2018. This included 9.2 million males and 5.3 million females ("Alochol Use Disorder", NIH). Adolescents can be diagnosed with AUD as well, with 401,000 teenagers from 12-17 years of age receiving a diagnosis of AUD in 2018 ("Alochol Use Disorder", NIH). There are over 88,000 alcohol-related deaths in the United States annually (Kranzler and Soyka, 2018). Total alcohol consumption per capita in people 15 years and older is projected to increase all over the world, making alcohol abuse very common and dangerous if not treated properly (Hammer et al., 2018).

2. Characteristics of AUD

Unhealthy alcohol use includes any alcohol consumption use that puts a person's health or safety at risk or causes alcohol-related problems ("Alochol Use Disorder-diagnosis and

treatment", Mayo Clinic). However, to be clinically diagnosed with AUD, individuals must meet several criteria from the DSM. The severity of the disorder is based on the number of the criteria met ("Alochol Use Disorder," NIH). Some of the criteria of AUD include inability to limit the amount of alcohol an individual consumes, presence of alcohol cravings, development of tolerance to alcohol, presence of withdrawal symptoms, and alcohol consumption interfering with one's daily lifestyle ("Alcohol Use Disorder-diagnosis and treatment," Mayo Clinic). There are several treatment options for AUD, such as psychiatric treatment (Alcoholics Anonymous, individual psychotherapy) or prescription drugs. However, AUD is a complex disease with many factors that can make it difficult to effectively treat. Consequently, treatment is frequently unsuccessful (Ilgen et al., 2006).

3. AUD is a complex disease

AUD is a multi-factorial disease, meaning there is both a genetic and environmental component to the disease (Reilly et al., 2017). The risk for developing AUD is therefore based on an individual's unique genetic composition and lifetime experiences (Litten et al., 2015). A twin and adoption study showed that 50-60% of phenotypic variations in AUD are due to a genetic component. There is also a 50% variance in the risk for AUD, and the age at the time of diagnosis can be attributed to genetic factors (Matsushita and Higuchi, 2014). Another study on the transition from alcohol abuse to dependence revealed that 26.6% of those with alcohol abuse eventually progress to dependence at some time in their lives. This is typically due to factors such as sociodemographic and psychopathological factors (Flórez-Salamanca et al., 2013). All these factors make it challenging to identify a set of universal genetic and environmental factors that drive the risk to develop AUD (Reilly et al., 2017).

4. Known genetic contributions influencing risk for AUD.

A large body of research has been done to examine environmental and genetic factors contributing to AUD to understand how people develop this disorder (Edenberg and Foroud, 2013). Multiple strategies have been used to identify genes and genetic pathways that may influence various aspects of alcohol abuse. Since the process of addiction involves hundreds of genes, only a few functional loci have been found to be associated with alcoholism (Ducci and Goldman, 2008). Also, the vast majority of studies were underpowered and individual genes tend to have small effects, making the unambiguous identification of associated genes very challenging. Two known genes that have been associated with alcohol abuse encode the alcohol metabolizing enzymes ALDH2 and ADH1B. These genes were the first genes to have been associated with alcoholism because their molecular mechanism involves breaking apart alcohol molecules in the liver, making its influence to alcoholism very convincing (Ducci and Goldman, 2008).

Multiple large population addiction studies using GWAS (Genome-Wide Association Studies) have revealed hundreds of genes that are associated with alcohol-related behaviors. A GWAS study of over 500,000 individuals was conducted from the Alcohol Use Disorder Identification Test (AUDIT). This test is a screening tool designed to identify hazardous alcohol use in the past year (Sanchez-Roige et al., 2019). This test consists of 10 items across three dimensions of alcohol consumption (AUDIT-C) and dependence and harmful alcohol use (AUDIT-P) (Sanchez-Roige et al., 2019). This study did a GWAS for both AUDIT-C and -P with a population from BioBank and 23andMe to identify associated risk loci. The top hits from this study include *CADM2, MAPT, FUT2, SLC39A8, JACB, LINC01833, ADH1C, ADH1B, KLB,* and *GCKR.*

These top hit genes had the strongest association with AUDIT scores and included the alcohol metabolism genes (Sanchez-Roige et al., 2019). This study concluded that AUDIT scores in certain population-based cohorts can be used to explore the genetic basis of both alcohol consumption and AUD (Sanchez-Roige et al., 2019).

Another GWAS study of tobacco and alcohol use using 1.2 million individuals was recently conducted. This study found 566 variants and 406 loci that are associated with multiple stages of alcohol and tobacco use (Liu et al., 2019). The top hits for alcohol use include genes such as *MEF2C, FUT1, CGKR, CADM2, ADH1A, MAPT, SPNS1, AUTS2.* They also found that alcohol phenotypes are negatively correlated with health conditions, and increased genetic risk for alcohol use is associated with lower disease risk (Liu et al., 2019). However, since substance use is embedded in a large complex of mechanisms and relationships between genes and environment, caution must be used in drawing any conclusions (Liu et al., 2019). The findings from this GWAS analysis represent a major step forward in understanding these multi-factorial addiction diseases (Liu et al., 2019).

A different GWAS study on problematic alcohol abuse (PAU) was done to identify risk genes of a population of over 400,000 European ancestry individuals (Zhou et al., 2020). This study identified 29 independent loci, with 19 of them being novel genes. Some of the genes from this list include: *PDE4B, GCKR, SIX3, VRK2, THSD7B, IRS1, CADM2, KLB, ADH1B, ADH1C, SLC39A8, DPP6, PENK, UNC5B, FUT2* (Zhou et al., 2020). The study also revealed that PAU was genetically correlated with 139 phenotypes, the top correlation being neuropsychiatric traits. Overall, this study was able to implicate numerous genes in alcohol abuse (Zhou et al., 2020).

All three of the studies reviewed here were interested in determining loci that were associated with various aspects of alcohol abuse. Some of the genes that were listed in all three of the individual studies include *FUT2, CADM2, KLB, ADH1B, ADH1C* and *GCKR,* strongly suggesting that these genes would be appropriate for further study. These three studies were also able to determine genetically correlated phenotypes such as a neuropsychiatric traits, consumption and abuse. By completing these large GWAS studies, we can use the results to study certain genes and pathways in model organisms to better understanding alcohol-related behaviors.

B. *Drosophila melanogaster* as a model for investigating behavioral responses to alcohol

1. Conservation between humans and flies

Model organisms have emerged as powerful genetic experimental platforms for investigating genes influencing alcohol-related behaviors and molecular mechanisms similar to those in humans. Specifically, the fruit fly, *Drosophila melanogaster* is a low-cost, fast-reproducing model organism used to study the genes associated with behavioral responses to alcohol (Grotewiel and Bettinger, 2015). Flies are used to study alcohol-related behavior because of the similar molecular mechanisms shared with humans, specifically, the major molecular machinery that controls the nervous system (Chan et al., 2015). Behavioral responses to ethanol are also conserved in humans and flies (Chan et al., 2015; Engel et al., 2019). Low doses of alcohol result in increased psychomotor activity in humans and locomotor behavior in flies, while higher doses result in sedation in both species (Grotewiel and Bettinger, 2015). Flies can also develop a tolerance and withdrawal symptoms to ethanol over time in a manner that is phenotypically similar to that in humans (Adkins et al., 2017). Consumption of ethanol can

result in any of the above behavioral changes in both humans and flies (Engel et al., 2019). There are a number of behavioral assays that can be performed on flies to measure their different types of behavioral responses to ethanol exposure.

2. Current approaches used to investigate genetic contributions to alcohol-related behavior in flies

Flies have multiple behavioral responses to ethanol including sedation, locomotor activation, withdrawal, tolerance, and consumption. Ethanol sedation is used to measure initial sensitivity of the fly toward ethanol exposure (Sandhu et al., 2015). Flies are exposed to ethanol vapor (this increases their internal ethanol as it would in humans when drinking) and then the number of flies that become sedated during the ethanol exposure is recorded. These raw data are then converted into a percent active as a function of time. The percent active can further be quantified as Sedation Time 50 (ST50, the time required for 50% of the flies to become sedated. (Sandhu et al., 2015) as a routine measure of ethanol sedation in flies. Longer ST50 values indicate resistance to sedation, whereas shorter ST50 values indicate increased sensitivity to sedation.

Chronic or repeated exposure of humans to alcohol can lead to the development of tolerance, defined as a blunted response during a prolonged or subsequent discrete exposure to the drug. Flies also develop tolerance to alcohol when measured with sedation, postural control or locomotor activation studies (Wolf et al., 2002). Flies also prefer to consume ethanol-containing food compared to food without ethanol and, importantly, this preference increases during repeated exposure of flies to food laced with ethanol. The two-choice Capillary Feeder (CAFE) assay is used to measure ethanol consumption and preference. Flies are allowed to feed

from two different capillaries, one with nonethanol liquid food and the other with liquid food containing ethanol. Ethanol preference is quantified by calculating a preference index (PI). The PI is defined by ethanol consumption [(ethanol consumption - nonethanol consumption)/total consumption]. The PI will vary between -1 and +1, positive values indicating ethanol preference and negative values indicating repulsion (Devineni and Heberlein, 2009). The CAFE assay models several features of human addiction including flies increasing ethanol consumption and preference overtime, voluntary consumption leading to pharmacologically relevant ethanol concentrations in the flies, flies overcoming an unpleasant stimulus to obtain ethanol, and flies exhibiting withdrawal symptoms when deprived from ethanol (Devineni and Heberlein, 2009).

In humans, the more we consume alcohol, the higher tolerance we build up to the physiological and behavioral response to a particular dose of alcohol (Scholz et al., 2000). Flies can exhibit a similar response when exposed to ethanol multiple times. Adult *Drosophila* can develop tolerance to the sedating and locomotor impairing effects of ethanol. Rapid tolerance is a phenomenon defined as a reduction in the intensity of the effects of ethanol upon repeated exposure (Berger et al., 2004). To measure rapid tolerance, flies are first exposed to ethanol to become sedated, are allowed a period of recovery in the absence of ethanol, and are then subjected to ethanol sedation a second time (Sandhu et al., 2015). An ST50 is calculated for both the initial exposure and for the second exposure to determine if the flies develop a tolerance to ethanol, evidenced by an increased ST50 during the second exposure.

Alcohol consumption can also have an effect on locomotor activity in flies. In order to observe fly locomotor activity, flies are exposed to ethanol vapor and video-recorded (Wolf et al., 2002). The motion of individual flies is then determined in a combination of computational

methods (Wolf et al., 2002). These methods quantify locomotion of flies in an automated matter. Ethanol affects flies by dynamically altering several parameters of walking behavior during the course of exposure. Acute ethanol exposure increases a fly's walking speed and turning, whereas prolonged exposure leads to loss of postural control and sedation (Wolf et al., 2002).

Flies can also develop withdrawal symptoms. Acute alcohol treatment impairs the performance of fly larvae in a simple associative learning and memory assay (Robinson et al., 2013). This assay can be used to measure signs of withdrawal (Robinson et al., 2013). Larvae are chronically fed alcohol-containing food, they acquire tolerance, and then behave as if they have not been exposed to alcohol. However, removal of the alcohol-containing food from the larvae resulted in withdrawal, and nervous system hyperexcitability (Robinson et al., 2013). This suggests that flies can adapt to become dependent on alcohol and show signs of withdrawal.

3. Genetic manipulations in flies

There are different strategies used to study the underlying genetic mechanisms that may influence a fly's behavioral response to alcohol. Broadly, there are two different general strategies to manipulate a fly's genome. The first is a transgenic strategy including the GAL4-UAS system and the GeneSwitch inducible expression system. The second strategy is to mutate endogenous genes in the fly genome. Mutations can be caused by chemical or other mutagens, chromosomal deletions, or transposon insertions. Both of these general strategies have proven successful in studying the role of fly orthologues of human genes that may influence AUD.

The GAL4 system is a method for ectopic gene expression that allows the activation of (in principle) any gene in different tissue and even cell-specific patterns. An advantage to this

system is the separation of the GAL4 protein from its target gene in distinct transgenic lines. This ensures that the target gene is largely silent except in the presence of GAL4 (Southall et al., 2008). The GAL4 system is highly flexible, providing a versatile tool for controlling ectopic expression both spatially and temporally. This system can be used to activate gene expression, express altered forms of proteins, and toxins for cell ablation or inhibition of cellular function (Duffy, 2002). It is also important to note that this system can be used to express RNAi (RNA interference).

GAL4 is a gene expressed by, identified in, and cloned from yeast, *Saccharomyces cerevisiae,* as a regulator of genes induced by galactose that has been well studied for over 30 years (Duffy, 2002; Southall et al., 2008). The 881-amino acid GAL4 protein can be separated into a DNA binding domain and an activation domain, which can function independently of each other. The GAL4 protein binds a 17-nucleotide sequence called the Upstream Activation Sequence (UAS). The separation of the GAL4 protein from the UAS-gene of interest in independent transgenic lines confers many advantages on the GAL4 system (Duffy, 2002; Southall et al., 2008) . For example, a single UAS-gene can be analyzed in multiple tissues at several different time points through the use of different GAL4 drivers, and a single GAL4 driver can be used to express several different UAS-transgenes in separate studies (Duffy, 2002; Southall et al., 2008). Since the UAS construct is largely silent in the absence of GAL4, viable transgenic lines encoding toxic or apoptotic proteins can be generated in order to further study. This ability to target gene expression to different tissues and cells makes this a powerful tool for studying not only development and physiology of the fly, but also the genetic mechanisms that underlay alcohol-related behaviors in flies.

Another transgenic strategy used to alter gene expression in *Drosophila melanogaster* is the GeneSwitch system (GS). This is an inducible gene expression system that uses a modified GAL4-UAS activation system (Osterwalder et al., 2001). This system was developed so transgenes could be expressed after post embryonic development, or in adult flies only (Osterwalder et al., 2001). Here, the GAL4 protein remains in an inactive conformation until the presence of the drug, mifepristone (RU486). The mifepristone allows the GAL4 protein to change from its inactive conformation to its active conformation, then the protein can bind to the UAS sequence to activate a gene of interest (or an RNAi). The mifepristone can be administered to the flies by either feeding or immersing the animals in a solution containing the drug (Nicholson et al., 2008). By varying the timing and dosage of drug being administered to the flies, the expression of the transgene can also be controlled. This system, similar to the GAL4-UAS system, can also be used to study the genetic mechanisms of alcohol-related behaviors at different stages of a fly's lifecycle, making this transgenic system a powerful tool for connecting developmental processes to, or dissociating them from specific behavioral responses to alcohol.

Another inducible transgenic strategy for studying genes in *Drosophila* is the tetracycline inducible system. This system is not as frequently used as the GeneSwitch inducible system, but still has a number of advantages (Stebbins et al., 2001). This system relies on the specific, high-affinity binding of the *Escherichia coli* Tet repressor protein (TetR) to the Tet operator *(TetO*). The tetracycline operator (*TetO)* is bound by either the tetracycline repressor protein (TetR) or by a tetracycline repressor mutant (rTA) (Osterwalder et al., 2001). When flies are exposed to doxycycline, the transgene is rapidly induced in adult flies, larvae, and embryos. It is also

possible to modulate gene induction by controlling the dosage of doxycycline in the food (Stebbins et al., 2001). These techniques are conceptually powerful tools, but have the practical limitation in *Drosophila* that new tissue specific TetR or rTA drivers as well as *tetO* responder lines need to be generated (Osterwalder et al., 2001). Although, this system can also be used to study the genetic mechanisms of alcohol-related behavior in flies, it was developed around the same time as GS, but it was the GeneSwitch inducible system that took off in the *Drosophila* field.

RNAi interference (RNAi) is an endogenous cellular mechanism triggered by expression of double stranded RNA (dsRNA), which leads to the degradation of homologous RNA molecules (Heigwer et al., 2017). Upon introduction of dsRNA, a complex including the DICER protein is formed that cuts the duplex RNAs into 21-nucelotide fragments. One strand of the dsRNA, called the guide strand, will bind an argonaute protein. The guide strand plus the argonaute protein will form a complex called RNAi-induced silencer complex (RISC). This complex will bind to the complementary mature mRNA strand and cleave that portion of mRNA, that portion is then degraded (Heigwer et al., 2017). This strategy will largely silence the target gene. In order to activate RNAi in *Drosophila melanogaster*, most RNAis are under UAS control. The RNAi can be expressed using the GeneSwitch or GAL4-UAS systems described above.

Mutations of genes are also another genetic manipulation used in *Drosophila.* Two main approaches are typically used: transposon insertions and chemical mutagenesis. Mutagenesis via insertions of engineered transposons can be used to analyze gene function and regulation. A transposon is a mobile DNA element that once inserted in the genome can disrupt gene

function and/or expression (Bellen et al., 2011). Transposons can also be remobilized to generate other useful mutations in the gene region where it resides through the processes of local jumping or imprecise excision (Bellen et al., 2011). Collections of insertion mutations have been created with genetic markers such as eye color, body color, drug resistance, etc. allowing multiple insertions to be manipulated (Bellen et al., 2004).

Chemical mutations have the major advantage of being unbiased and sometimes permitting the generation of allelic series. Chemical mutagenesis with ethyl methane sulfonate (EMS) is a powerful approach for generating mutant strains of cells or organisms for studying all biological processes. In *Drosophila,* a causative DNA lesion for a specific phenotype can be identified by mapping of the mutant locus using visible genetic markers that span the genome (Cingolani et al., 2012). All mutation strategies and transgenic strategies described can be used to manipulate *Drosophila* orthologs of human genes known to be associated with AUD.

4. Genes known to influence alcohol-related behaviors in Drosophila melanogaster

Multiple studies in flies have significantly contributed to our understanding of molecular-genetic mechanisms that influence alcohol-related behaviors (Grotewiel and Bettinger, 2015). Approximately 150 genes in flies with roles in behavioral responses to alcohol have been described, however only a few genes will be discussed here. These genes have been highly studied in flies to make them candidate genes that may influence AUD in humans. These include: Insulin signaling pathway genes, *AUTS2* ortholog, Heat shock proteins, the *XRCC5* gene and the gene at the core of my thesis project, *Mef2.*

The molecular genetic analysis of flies have demonstrated the existence of the extremely well conserved insulin signaling pathway (Garofalo, 2002). The role of insulin

signaling in the control of lifespan, reproduction, and metabolism has also been revealed by genetic studies of this pathway in flies. Studies using loss of function (LOF) mutants in the *InR* genetic pathway (fly ortholog pathway for insulin signaling) have revealed that this pathway in the nervous system influences sensitivity to the intoxicating effects of ethanol (Corl et al., 2005). Studies on the insulin signaling pathway in flies suggests an evolutionarily conserved role in regulating the response of the nervous system to intoxicating drugs, giving a possible candidate pathway to study more in depth in other model organisms and humans to understand the genetics of AUD (Corl et al., 2005).

Flies have also been used to explore the role of *tay*, the fly ortholog gene to human *AUTS2*. A single nucleotide polymorphism (SNP) in the intron region of *AUTS2* was found in a GWAS study looking at alcohol consumption(Engel et al., 2019). *AUTS2* was also studied when comparing mice lines bread for alcohol preference (HAP1) with low alcohol preferring mice (LAP1) (Mulligan et al., 2006; Schumann et al., 2011; Engel et al., 2019). Reduced expression of the fly ortholog, *tay,* a regulator of the epidermal growth factor (EGFR) (Engel et al., 2019), decreased ethanol sensitivity (Schumann et al., 2011). The genetic studies in flies suggest that the *InR* and *tay/AUTS2* genes might influence AUD in human via regulation of ethanol sedation sensitivity.

Microarray experiments have been used in the past to determine changes in gene expression following ethanol exposure in flies. One of the changes seen in these experiments involves the regulation of genes encoding heat shock proteins (HSP). Out of these genes, *hsp26* is the most strongly regulated HSP-encoding genes (Awofala et al., 2011). *hsp26* encodes an HSP that has been shown to be temperature-regulated in yeast and regulates aging and

oxidative stress in flies (Awofala et al., 2011). Previous research has demonstrated that *hsp26* is required for development of tolerance to ethanol. Further studies of the role in *hsp26* in the nervous system of flies could provide more information for understanding the development of tolerance and dependence in alcoholics (Awofala et al., 2011). Investigating the pathway of *hsp26* could ultimately lead to a better understanding of genes involved in tolerance and potentially AUD.

Other genes that are known to influence ethanol-induced behaviors in flies include *Icarus (ics)*, which encodes *Ras suppressor 1 (Rsu1)* (Ojelade et al., 2015), and *Mef2* (myocyte-enhancer factor 2), a MADS-box transcription factor (Potthoff and Olson, 2007) (see next section). When genetic manipulations are made to either of these genes, there is a significant change in the fly's behavioral response to ethanol. *Drosophila* lacking *Rsu1* show reduced sensitivity to ethanol-induced sedation. A more recent study did a GWAS of genes that are associated with alcohol susceptibility in humans and found the *XRCC5* gene. Further testing with the *Drosophila melanogaster* ortholog, *Ku80*, found that when flies were exposed to ethanol their initial sensitivity significantly decreased in *Ku80* mutants (Juraeva et al., 2015). There are over 150 other *Drosophila* genes that are also known to influence ethanol-induced behavioral responses, and they all play a part in understanding the genetic mechanisms underlying AUD.

C. Myocyte Enhancer Factor 2 (*Mef2*)

1. Mef2 *in humans and Drosophila melanogaster*

Mef2 (myocyte enhancer factor-2) encodes a family of transcription factors with a variety of different functions. MEF2 proteins belong to the evolutionarily ancient MADS family

(Potthoff and Olson, 2007). There are four mammalian genes*, Mef2 A, B, C* and *D*. Of the four mammalian genes, *Mef2A, C,* and *D* can be alternatively spliced to produce transcripts and proteins which may have significantly different functions (Brand, 1997). These different functions could play a role in development of skeletal, cardiac, and smooth muscle cells (Black and Olson, 1998). MEF2 proteins bind to consensus DNA sequence YTA(A/T)$_4$TAR as homo-or heterodimers.

The MEF2 protein family plays central roles in a variety of physiological processes including development of the nervous system and muscle development (Pon and Marra, 2016; Chen et al., 2017). This family is also implicated in human diseases such as liver fibrosis, cancers, and neurodegenerative diseases (Chen et al., 2017). For example, MEF2B and D are involved in maintaining Epstein-barr virus (EBV), which is known as one of the nine human herpesvirus types and drives resting B cells to continuously proliferate infected cells (Kempkes and Robertson, 2016). EBV infection can be linked to several malignancies including Burkitts' lymphoma, Hodgkin's lymphoma, gastric carcinoma, and many more human diseases (Kempkes and Robertson, 2016).

In *Drosophila melanogaster,* there is only one orthologue of *Mef2* gene. Fly *Mef2* is critical for the differentiation of muscle cell lineages and is essential for viability of the flies (Crittenden et al., 2018). *Mef2* is also essential for the formation of mushroom bodies in the embryonic brain and for normal development of wings in adults. *Mef2* also transmits clock information to machinery involved in neuronal remodeling, which contributes to locomotor activity rhythms and circadian behavior (Sivachenko et al., 2013).

2. Mef2 *influences ethanol sedation in Drosophila melanogaster*

Previous studies indicate that low initial sensitivity to alcohol may be a risk factor for later alcohol abuse. A 12 question alcohol form called "The Self-Rating Effects (SRE) of alcohol" was developed to help predict if a person has a low response to alcohol (Schuckit et al., 1997). Dr. Alexis Edwards conducted a meta-analysis of two population-based genome-wide association studies (GWAS) of SRE. This analysis interrogated over 18,000 genes, 37 of which had a nominally significant p value ($p_{gene}<0.001$) for SRE. Of those 37 genes, 29 had orthologues in *Drosophila* (Schmitt et al., 2019). Nine human genes were selected for further testing in *Drosophila* based on previous research suggesting they might be related to alcohol use or misuse.

The human genes *APP, ATG5, BORCS8, MEF2B, GPD2, ISL1, MEF2B, PCDH15,* and, *SFSWAP* are orthologs to 12 *Drosophila* genes. RNAi reagents were obtained from public stock centers to manipulate the most conserved fly genes (Schmitt et al., 2019). These genes were tested by expressing RNAi targeting each gene in neurons using *elav*-GAL4 and assessed ethanol sedation in adults. Three RNAi transgenes against *Mef2* (two with the same target sequence) increased ST50 values compared to the genetic controls (Schmitt et al., 2019). These results led us to believe that other genes associated with *Mef2* may likewise play a role in ethanol sedation.

D. Significance

As mentioned in section A, AUD has many negative consequences on people all over the globe. This includes premature death or preventable death, mental health effects, decrease in productivity and the development of disease states (Edenberg and Foroud, 2013). Current

treatment options include individual or group psychotherapies and some prescription drug therapies (Kranzler and Soyka, 2018). However, some patients who initially achieve abstinence from drinking will eventually relapse to alcohol consumption within a year of receiving treatment (Johnson and Ait-Daoud, 1999). Although these are viable treatment options, there is still a large gap in the knowledge of understanding and treating AUD.

AUD is a complex disease with both an environmental component and a genetic component. *Drosophila melanogaster* is a powerful tool for investigating the biology of genes implicated in human AUD. Additionally, flies can be used to identify novel candidate genes for roles in human AUD. These studies will not only help us to understand the molecular and genetic mechanisms of this disease, but these mechanisms studied in flies can eventually be translated back to humans to further study. Researchers will then be able to better diagnose and potentially treat patients with AUD.

Chapter 2-Materials and Methods

A. Fly husbandry and stocks

Flies were cultured in an environmental chamber with a 12-hour light/dark cycle. They were maintained at 25^{o}C and 60-65% relative humidity. Flies were grown on *Drosophila* food medium containing 10% sugar, 3.3% cornmeal, 2% yeast, 1% agar, 0.1 g/L ampicillin, 0.125 g/L chloramphenicol, 2 g/L tegosept, 0.02 g/L tetracycline, and live yeast.

w[A] (stock #5905), transposon *spin* mutant stocks, *spin* RNAi (stock #27702), *ph-d* (stock #31190), and GeneSwitch (GS) lines were all obtained from Bloomington *Drosophila* Stock center (BDSC, Bloomington, IN). *w*[VDRC], and *Mef2* RNAi (stock #v15550) were obtained from the Vienna *Drosophila* RNAi center (VDRC, Vienna, Austria). All RNAi lines, transposon, GS, and other lines are listed in Table 1.

Genotype	Stock #	Description	Source
w[1118]	5905	lab control strain	BDSC
elav-Gal4 w[A]/FM7B	NA	neuronal driver	P. Bhandari
w[VDRC]	NA	VDRC control strain	VDRC
w[1118][A]; CyO/Sp[A]; 3[A]	NA	balancer	Lauren Thomas
w[1118];2]VDRC];v15550[VDRC]	v15550	*Mef2* RNAi	VDRC
y[1] v[1]; P{y[+t7.7] v[+t1.8]=TRiP.JF02782}attP2	27702	*spin* RNAi	BDSC
y[1] v[1]; P{y[+t7.7] v[+t1.8]=TRiP.JF01705}attP2	31190	*ph-d* RNAi	BDSC
y1 v1; P{TRiP.HMS02042}attP40	40875	*tau* RNAi	BDSC
y1 v1; P{TRiP.HM05101}attP2	28891	*tau* RNAi	BDSC
w1118; P{GD8682}v25023	v25023	*tau* RNAi	VDRC
w1118; P{GD8682}v25024	v25024	*tau* RNAi	VDRC
w1118; P{GD10150}v25666	v25666	*tau* RNAi	VDRC
w1118; P{GD10150}v25667	v25667	*tau* RNAi	VDRC
P{KK109359}VIE-260B	v101386	*tau* RNAi	VDRC
P{TRiP.HMS00445}attP2	32447	*Frl* RNAi	BDSC
w1118; P{GD10799}v34412	v34412	*Frl* RNAi	VDRC
w1118; P{GD10799}v34413	v34413	*Frl* RNAi	VDRC
P{KK101703}VIE-260B	v110438	*Frl* RNAi	VDRC
PBac{fTRG01118.sfGFP-TVPTBF}VK00002	v318758	*Frl* RNAi	VDRC
w1118; P{GD10457}v21446	v21446	*Frl* RNAi	VDRC
P{KK110613}VIE-260B	v102402	*CG6770* RNAi	VDRC

w1118; P{GD13710}v35825	v35825	*CG6770* RNAi	VDRC
y[1] v[1]; P{y[+t7.7] v[+t1.8]=TRiP.JF02715}attP2	27561	*CG6770* RNAi	BDSC
w1118 P{GD1650}v3229	v3229	*spin* RNAi	VDRC
w1118; P{GD1650}v46030	v46030	*spin* RNAi	VDRC
P{KK104813}VIE-260B	v105462	*spin* RNAi	VDRC
P{TRiP.GL01585}attP2	43975	*chrb* RNAi	BDSC
P{KK109191}VIE-260B	v105757	*chrb* RNAi	VDRC
PBac{fTRG00853.sfGFP-TVPTBF}VK00002	v318693	*chrb* RNAi	VDRC
w1118; P{GD9925}v25506	v25506	*Scyl* RNAi	VDRC
w1118; P{GD9925}v25504/CyO	v25504	*Scyl* RNAi	VDRC
y1 v1; P{TRiP.HMJ02221}attP40	42564	*stv* RNAi	BDSC
w1118; P{GD10796}v34408	v34408	*stv* RNAi	VDRC
w1118; P{GD10796}v34409/TM3	v34409	*stv* RNAi	VDRC
y1 v1; P{TRiP.JF01392}attP2	31608	*ph-p* RNAi	BDSC
w1118; P{GD4480}v10679	v10679	*ph-p* RNAi	VDRC
P{KK108787}VIE-260B	v100811	*ph-p* RNAi	VDRC
y[1] sc[*] v[1] sev[21]; P{y[+t7.7] v[+t1.8]=TRiP.GL00083}attP2/TM3, Sb[1]	35207	*ph-p* RNAi	BDSC
y[1] sc[*] v[1] sev[21]; P{y[+t7.7] v[+t1.8]=TRiP.HMS00082}attP2	33669	*ph-p* RNAi	BDSC
P{TRiP.HMS05292}attP40	63018	*ph-d* RNAi	BDSC
P{VSH330529}attP40	v330529	*ph-d* RNAi	VDRC
Tubulin GS	NA	ubiquitous GS driver	Scott Pletcher
P{w[+mW.hs]=Switch2}GSG3315-1, w[*]	59949	putative neuronal GS	BDSC
y[1] w[*]; P{w[+mW.hs]=Switch2}betaTub56D[GSG5793]/CyO	40333	putative neuronal GS	BDSC
w[*]; P{w[+mW.hs]=Switch2}GSG3763	40286	putative neuronal GS	BDSC
P{w[+mW.hs]=Switch2}GSG5970, w[*]	40251	putative neuronal GS	BDSC
w[*]; P{w[+mW.hs]=Switch2}GSG4948	40294	putative neuronal GS	BDSC
w[*]; P{w[+mW.hs]=Switch2}GSG4784/CyO	40283	putative neuronal GS	BDSC
y[1] w[*]; P{w[+mC]=elav-Switch.O}GSG301	43642	putative neuronal GS	BDSC
w[1118]; P{w[+mC]=MB-Switch}3/TM6C, Sb[1]	81013	putative neuronal GS	BDSC
P{w[+mGT]=GT1}*ph-d*[BG02139] w[1118]	12551	*ph-d* transposon	BDSC
Mi{ET1}*ph-d*[MB01363] w[1118]	23076	*ph-d* transposon	BDSC
y[1] w[67c23]; P{w[+mC] y[+mDint2]=EPgy2}*spin*[EY08566]	16431	*spin* transposon	BDSC
y[1] w[67c23]; P{w[+mC] y[+mDint2]=EPgy2}*spin*[EY10097]	19895	*spin* transposon	BDSC
y[1] w[67c23]; P{w[+mC]=lacW}*spin*[k09905]/CyO	10948	*spin* transposon	BDSC
w[*]; P{w[+mC]=UAS-TeTxLC.tnt}G2	28838	UAS-Tetanus Toxin light chain	BDSC
y[1] w[*]; P{w[+mC]=UAS-GFP::lacZ.nls}30.1	6452	UAS-*LacZ* with nuclear localization signal	BDSC
Nrv2-Gal4:UAS-GFP	NA	neuronal UAS-GFP	Paul Salvaterra

Table 1: Fly stocks used.

B. Backcrossing

All transposon lines (stocks #12551, 23076, 10948, 19895, 16431) for *spin* and *ph-d* were backcrossed to *w*[A] for seven generations to standardize the genetic background. Flies with red eyes or green eyes (*ph-d* stock #23076) were selected for the next generation in the backcross. All *spin* lines (stocks # 16431, 10948, 19895) were balanced to *CyO/Sp w*[A].

C. Ethanol sedation experiments

Flies were collected in groups of 11 by a single sex under brief CO_2. Collected flies recovered overnight in upside-down, non-yeasted food in the environmental chamber (25^{o}C, 60-65% humidity). Fresh 85% ethanol was made no more than one week before the experiments and all ethanol sedation studies were done at 20-23^{o}C with relative humidity between 55-65% and standard laboratory lighting. To initiate ethanol sedation studies, flies were transferred to empty food vials and sealed with a cotton Flug. Sedation time 50 (ST50) values, the time it takes for 50% of the flies in the individual vials to become sedated, was determined by exposing the flies to 85% ethanol vapor and assessing the number of sedated flies at 6-min intervals. All vials were tested in groups of four, by pouring the 85% ethanol every five seconds within each group and testing one group per min over a period of six min as described (Chan, 2013; Sandhu et al., 2015). The experimenter was blind to all genotypes during the experiments.

D. Immunohistochemistry and imaging

Brains from adult females flies for desired genotypes were dissected in PBT (100 mM Phosphate buffer, pH 7.2, with 0.03% (v/v) triton X-100) under a dissecting microscope. Brains were then fixed in 1.5 mL snap cap tubes containing 4% paraformaldehyde for 20 min at room temperature on a rotator. Fixed brains were washed three times with PBT and blocked with 5%

normal goat serum (NGS). To assess *spin* expression, brains were incubated in primary antibody (5% NGS, mouse anti-elav and rabbit anti-SPNS2) for 48 hours at 4^{o}C on a rotator. Brains incubated in the primary antibody serum were then washed three times with PBT and incubated in secondary antibody serum (5% NGS, chicken-anti Rabbit, Alexa 647, Goat anti-mouse, Alexa 488) for another 48 hours at 4^{o}C on a nutator. Brains were then washed three times with PBT and mounted onto microscope slides using SlowFade (Invitrogen by Thermo Fisher scientific EU, Oregon). This follows the protocol as described (Wu and Luo, 2006). Images of the brains were captured and collected using a Zeiss LSM 700 confocal microscope (Carl Zeiss Microscopy, LLC, Thornwood, NY) housed in the VCU Department of Anatomy and Neurobiology Microscope Facility. All images were taken using a 10X objective with a numerical aperture of 0.3, with a pin hole of one Airy disc Unit. The microscopy settings were optimized from control flies (*spin* RNAi transgene control flies). The same settings (gain, offset, power) were used in all images of different genotypes. All images were processed using Image J (National institute of health; Bourne and Bourne, 2010).

E. PCR and sequencing

Standard PCR methods was used for all experiments. All PCR reactions contained dNTPs, Dream Green Taq buffer, and Dream Taq polymerase according to the manufacturer's instructions (thermoscientific, Waltham, MA). The BioRad c1000 thermocycler (BioRad, Hercules, CA) was used for all PCR amplification with a temperature gradient (70.8, 68.7, 65.6, 61.7 ^{o}C). The PTC-100 programmable thermal controlled (MJ Research Inc., San Francisco, CA) was used for all other PCR amplification reactions. Primers used for each PCR reaction are listed

in Table 2. GeneWiz (South Plainfield, NJ) and SnapGene Viewer (Chicago, IL) was used for sequencing and analysis.

transposon	sense/antisense	sequence 5' to 3'	product length (estimate)
EY10097	fwd	GAG TTC CCG TTT CTC TGT GTG C	234 bp
	rev	TGT CCG TAT CCG AGG GCA TC	172 bp
k09905	fwd	GTG TGT CAT TGT TGT TGC TGC TGC	163 bp
	rev	CCT CGT CAA TAA CTG TAA GCC GAT C	194 bp
EY08566	fwd	TGC CGG CGA TAA GCG AAC C	332 bp
	rev	CAC TCA AGA ATT TCG GGA GCA AAC	175 bp
P element	--	CGACGGGACCACCTTATGTTATTTCATCATG	--

Table 2: Primers used in section 3 of results (chapter 3).

F. Statistical analyses

All statistical analyses were done on GraphPad Prism (GraphPad Software, San Diego, CA). Bonferroni multiple comparisons, one-way ANOVA, and unpaired T-tests were used as appropriate in accordance with the experimental design.

Chapter 3-Genetic Analysis of *spin*, a candidate gene downstream of *Mef2*

A. RNAi-based screen of Mef2-bound genes for a role in ethanol sedation

Introduction

The *Drosophila* ortholog to *MEF2B*, which is shown to be associated with SRE, is *Mef2* (myocyte enhancer factor-2). *Mef2* belongs to a family of MADS-box transcription factors (MCM1, agamous, deficiens, serum response factor). These transcription factors play multiple roles in myogenesis and morphogenesis in *Drosophila melanogaster* (Black and Olson, 1998). Central to my thesis project, *Mef2* plays a significant role in ethanol sedation (Schmitt et al., 2019). This led us to hypothesize that genes bound by Mef2 might also impact ethanol sedation.

A previous study of alcohol tolerance in *Drosophila* was interested in genes that change a fly's response to ethanol. The researchers screened a number of genes and found that flies with altered *Hr38* had a statistical significant change in ethanol tolerance and preference (Adhikari et al., 2019). The researchers also found that mammalian homologs of *Hr38* are transcriptionally regulated by Mef2. From there they wanted to know if ethanol upregulates *Hr38* through *Mef2* to promote an ethanol-response phenotype. They found that ethanol activates *Mef2* to induce *Hr38* (Adhikari et al., 2019). Previous studies in our lab found that *Mef2* RNAi knockdown in neurons as well as mutations in *Mef2* made flies resistant to ethanol sedation without a change in ethanol rapid tolerance (Schmitt et al., 2019). Since Mef2 binds 342 genes (Sivachenko et al., 2013) and the lab's previous experiments demonstrate that *Mef2* influences ethanol sedation, we hypothesized that one or more genes regulated by Mef2 may also influence ethanol sedation. The goal of my project was to begin testing this hypothesis.

Results

Identifying Mef2-bound genes related to alcohol-related behavior in flies

A GSCAN (GWAS & Sequencing Consortium of Alcohol and Nicotine use) of the 342 genes bound by Mef2 in flies was done to determine if any of the human orthologues of these genes are associated with gene expression changes related to alcohol consumption. Of those 342 genes, 15 had evidence of being nominally associated with gene expression related to alcohol consumption (Baccanu, unpublished). I was able to identify 36 RNAi transgenes targeting nine of those genes in flies (Table 3). I then determined whether neuronal expression of the RNAis targeting the nine genes led to an ethanol sedation phenotype in flies.

Testing Mef2-bound genes in ethanol sedation

I used the *elav*-Gal4 driver to express the RNAi transgenes in neurons and then determined whether expression of the RNAi transgenes influenced ethanol sedation. Flies expressing an RNAi transgene targeting *spin* (JF02782) were resistant (i.e. had higher ST50 values, red bar) than control flies with the *elav*-Gal4 driver (black bar) or the JF02782 RNAi transgene alone (grey bar) (Figure 1A, B). I confirmed these results by repeating the experiment with independent crosses and obtaining similar results (Figure 2A, B). Expression of other RNAi transgenes targeting *spin* in neurons led to no definitive effects on ST50 values, although there was a trend for *spin* v3229-expressing flies to be resistant to ethanol sedation (Table 3). Similar to the results with *spin* RNAi transgenes, expression of one RNAi targeting *ph-d* (JF01705) made flies resistant to ethanol sedation compared to genotype controls (Figure 1C, D), whereas expression of two additional *ph-d* RNAi transgenes had no consistent effect on ST50 values (Table 3). Neuronal expression of RNAi targeting the other genes (*CG6770, chrb, Frl, ph-p, scyl,*

stv and tau) had no consistent effects on ethanol sedation in flies relative to control strains (Table 3). Thus, of the 36 RNAi transgenes tested, one targeting *spin* and one targeting *ph-d* altered ST50 values.

gene	group	RNAi	ST50 (min)	statistical analysis	ST50 P value
spin	RNAi-expressing	JF02782	48.09	ANOVA	0.0002
	control	JF02782	30.65	BMC	0.0002
	control		34.6	BMC	0.0022
spin	RNAi-expressing	KK104813	37.44	ANOVA	0.2319
	control	KK104813	36.11	BMC	
	control		33.15	BMC	
spin	RNAi-expressing	v3229	39.01	ANOVA	0.0287
	control	v3229	33.23	BMC	0.1011
	control		31.2	BMC	0.0215
spin	RNAi-expressing	v46030	37	ANOVA	0.0135
	control	v46030	36.48	BMC	> 0.9999
	control		31.83	BMC	0.0148
ph-d	RNAi-expressing	JF01705	53.35	ANOVA	0.0009
	control	JF01705	43.03	BMC	0.0205
	control		37.21	BMC	0.0005
ph-d	RNAi-expressing	HMS05292	34.61	ANOVA	0.0002
	control	HMS05292	26.35	BMC	0.0011
	control		36.03	BMC	0.9921
ph-d	RNAi-expressing	VSH330529	38.03	ANOVA	0.0001
	control	VSH330529	27.2	BMC	< 0.0001
	control		34.18	BMC	0.1574
CG6770	RNAi-expressing	KK110613	28.01	ANOVA	0.3096
	control	KK110613	25.53	BMC	
	control		28.73	BMC	
CG6770	RNAi-expressing	v35825	34.43	ANOVA	0.4926
	control	v35825	35.8	BMC	

	control		33.05	BMC	
CG6770	RNAi-expressing	JF02715	35.33	ANOVA	0.4957
	control	JF02715	33.45	BMC	
	control		33.1	BMC	
Frl	RNAi-expressing	KK101703	31.38	ANOVA	0.7154
	control	KK101703	41.61	BMC	
	control		32.45	BMC	
Frl	RNAi-expressing	HMS00445	43.35	ANOVA	0.0507
	control	HMS00445	38.31	BMC	
	control		36.05	BMC	
Frl	RNAi-expressing	v34413	29.75	ANOVA	0.0007
	control	v34413	36.11	BMC	0.0011
	control		30.16	BMC	> 0.9999
frl	RNAi-expressing	v34412	37.79	ANOVA	0.2995
	control	v34412	38.15	BMC	
	control		35.21	BMC	
chrb	RNAi-expressing	KK109191	33.01	ANOVA	0.1162
	control	KK109191	26.66	BMC	
	control		32.73	BMC	
chrb	RNAi-expressing	GL01585	32.61	ANOVA	0.7009
	control	GL01585	30.36	BMC	
	control		32.05	BMC	
tau	RNAi-expressing	v25667	*25.78*	ANOVA	0.7108
	control	v25667	27.34	BMC	
	control		28.08	BMC	
Tau	RNAi-expressing	v25024	27.5	ANOVA	0.0564
	control	v25024	32.04	BMC	
	control		29.18	BMC	
Tau	RNAi-expressing	v25666	29.46	ANOVA	0.0305
	control	v25666	34.9	BMC	0.018
	control		32.14	BMC	0.343

tau	RNAi-expressing	KK109359	31.52	ANOVA	0.0091
	control	KK109359	42.73	BMC	0.0088
	control		34.2	BMC	0.8974
tau	RNAi-expressing	v25023	lethal		
	control	v25023			
	control				
tau	RNAi-expressing	HMS02042	22.29	ANOVA	< 0.0001
	control	HMS02042	21.75	BMC	> 0.9999
	control		31.54	BMC	0.0002
tau	RNAi-expressing	HM05101	27.85	ANOVA	0.1129
	control	HM05101	25.58	BMC	
	control		27.63	BMC	
scyl	RNAi-expressing	v25506	32.24	ANOVA	0.1344
	control	v25506	32.95	BMC	
	control		29.49	BMC	
stv	RNAi-expressing	HMJ02221	26.56	ANOVA	0.0236
	control	HMJ02221	28.85	BMC	0.0144
	control		32.41	BMC	0.515
stv	RNAi-expressing	v34409	36.63	ANOVA	0.2925
	control	v34409	38.56	BMC	
	control		38.94	BMC	
stv	RNAi-expressing	v34408	33.93	ANOVA	0.5855
	control	v34408	34.28	BMC	
	control		35.68	BMC	
ph-p	RNAi-expressing	JF01392	42.34	ANOVA	0.0354
	control	JF01392	41.45	BMC	> 0.9999
	control		35.61	BMC	0.0349
ph-p	RNAi-expressing	G1884	48.76	ANOVA	0.0107
	control	G1884	43.14	BMC	0.0914
	control		39.95	BMC	0.0064

ph-p	RNAi-expressing	GL00083	43.26	ANOVA	0.0009
	control	GL00083	39.84	BMC	0.17
	control		34.85	BMC	0.0005
ph-p	RNAi-expressing	v10679	38.73	ANOVA	0.2251
	control	v10679	34.15	BMC	
	control		36.23	BMC	

Table 3: Mef2-bound gene screen using ethanol sedation. All RNAis for the nine Mef2-bound genes are under UAS control and expressed using *elav*-GAL4. One-way ANOVA and Bonferroni multiple comparisons (BMC) were used to analyze ST50 results.

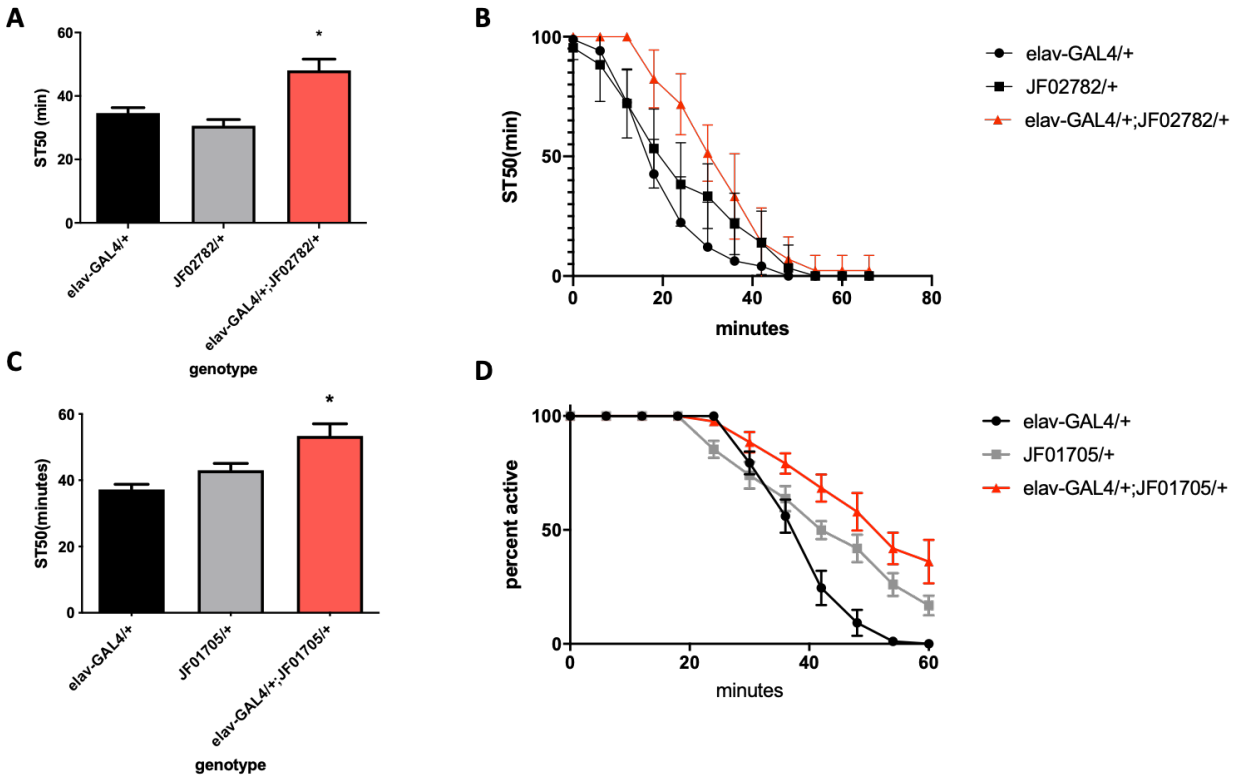


Figure 1: Ethanol sedation levels in flies with pan-neuronal expression of *spin* RNAi (A and B) and *ph-d* RNAi (C and D). B) and D) ethanol sedation time courses of flies with the pan-neuronal expression of *spin* C) and *ph-d* D) RNAis. A) and C) ST50 values derived from time courses in panels B and D. *Bonferroni multiple comparisons (BMCs), p=0.0002 (A), p=0.005 (C); n=8.

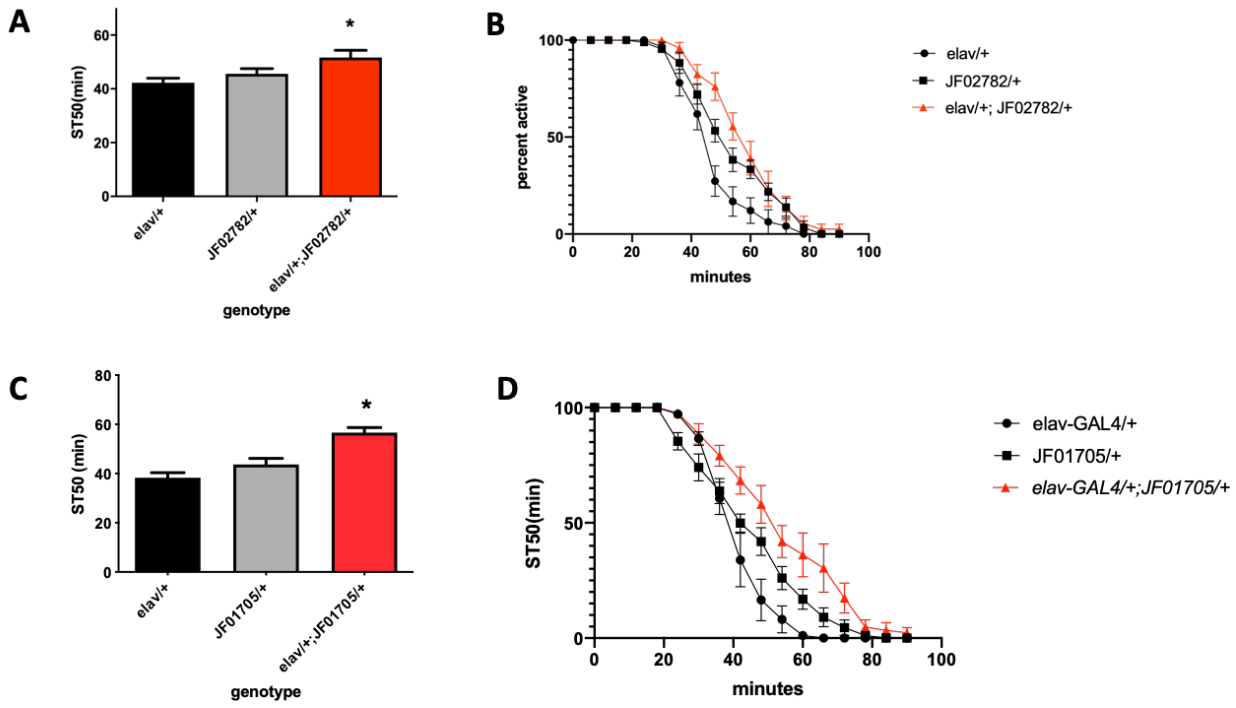


Figure 2: Ethanol sedation levels in flies with pan-neuronal expression of *spin* RNAi (A and B) and *ph-d* RNAi (C and D) repeated for confirmation. B) and C) ethanol sedation time courses of flies with the pan-neuronal expression of *spin* C) and *ph-d* D) RNAis. A) and C) ST50 values derived from time courses in panels B and D. *Bonferroni multiple comparisons (BMCs), p=0.0162 (A), p=0.0101 (c); n=8.

Discussion

spin and *ph-d* were two genes that showed a significant change in ethanol sedation when the RNAi was neuronally expressed. Based on the results of this ethanol sedation screen *spin* and *ph-d* were chosen as candidate in ethanol sedation. *spin* (*spinster*) is a late endosome/lysosome membrane protein that works as a lysosomal sugar carrier (Kim et al., 2017). *ph-d* belongs to a locus of *Drosophila* called *polyhomeotic* (*ph*) and is a complex locus essential for the maintenance of segmenting flies during development (Hodgson et al., 1997). The *ph* group is split into proximal (*ph-p*) and distal (*ph-d*) transcription units that are regulated differently at the mRNA level during development as shown in previous research (Hodgson et al., 1997). However, *ph-d* was dropped as a candidate gene for ethanol sedation due to its complex locus and lack of reagents for further experiments. This leaves *spin* as the Mef2-bound gene as a candidate in ethanol sedation in *Drosophila melanogaster.*

B. Effects of *spin* transposon insertions on ethanol sedation

Introduction

Genetic mutations in genes are essential tools for analyzing gene function. The *Drosophila* Gene Distribution project (GDP) has been making mutations in genes all over the *Drosophila* genome. One class of mutations they make in different genes is inserting engineered transposable elements (transposons) (Bellen et al., 2004). These transposons are inserted within genes to disrupt gene expression, and because of this, these insertions into the genome are one of the most versatile approaches to manipulating *Drosophila* genes on a genome-wide scale (Bellen et al., 2004).

There are many categories of these transposable elements, one of these categories are "P transposable elements" (P-element transposons). These are one of the best-studied mobile DNA elements. The overall structure of these P-elements in *Drosophila* contain 31 bp terminal inverted repeats (TIR) at the ends of the transposon (Bellen et al., 2011). P-elements can also move around the genome with the help of the transposase enzyme within the P-element, however, the engineered P-elements from GDP do not contain this enzyme. Instead, these elements contain a "mini-white marker." This is a gene that turns the flies eyes red in a mutant background that otherwise would have white eyes. (Bellen et al., 2011) (figure 3). Since these transposable elements are so versatile, the goal of this project is to determine if the P-elements within the *spin* gene can influence ethanol sedation.

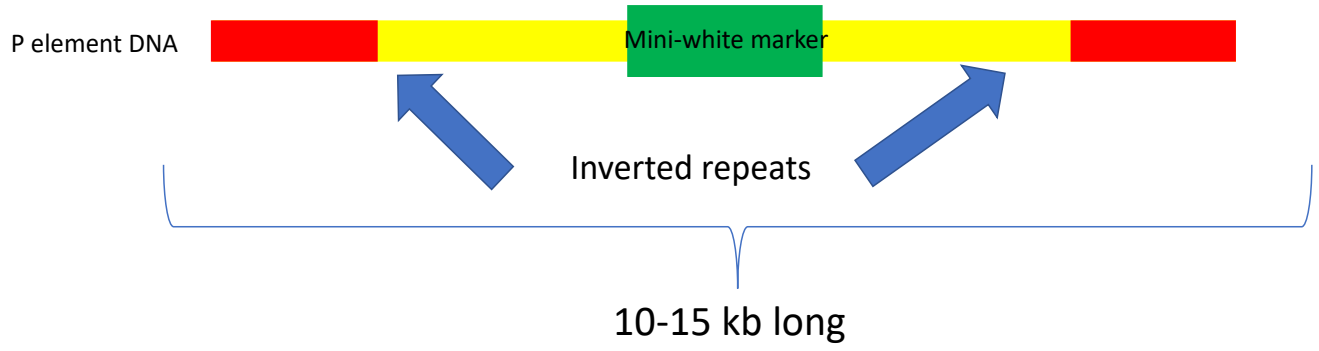


Figure 3: P element transposable element (P element). The red regions at either end of the transposon DNA are inverted repeats (~31bp in length). These repeats are the same on either side of the P element. These P elements contain a gene called “mini-white” which turns a fly’s eye red in a white mutant background; this is how transposons are tracked at the macroscopic level. These transposons can be anywhere from 10 to 15 kb long.

Results

Genotyping before backcross

Flies that contained the P-element in the *spin* gene were backcrossed so all flies had the same genetic background. All flies were genotyped for the P-element transposon using PCR with DNA that came from flies before entering the backcross and after finishing the backcross. Genotyping using PCR involved designing primers that targeted the DNA upstream of the transposon DNA and downstream of the transposon (figure 4). A primer was also designed to target the inverted repeats on either end of the transposon (P-element primer). For each P-element containing fly, two sets of reactions using different primer combinations were performed (figure 4). The first reaction was using the primer that targeted the DNA upstream of the transposon (forward primer) with the P-element primer (front end reaction; figure 4), and the second reaction used the primer targeting the DNA downstream of the transposon with the P-element primer (backend reaction; figure 4). A series of PCR reactions on the primers used and the transposon DNA was done to verify that the results from DNA gel electrophoresis were for the intended DNA.

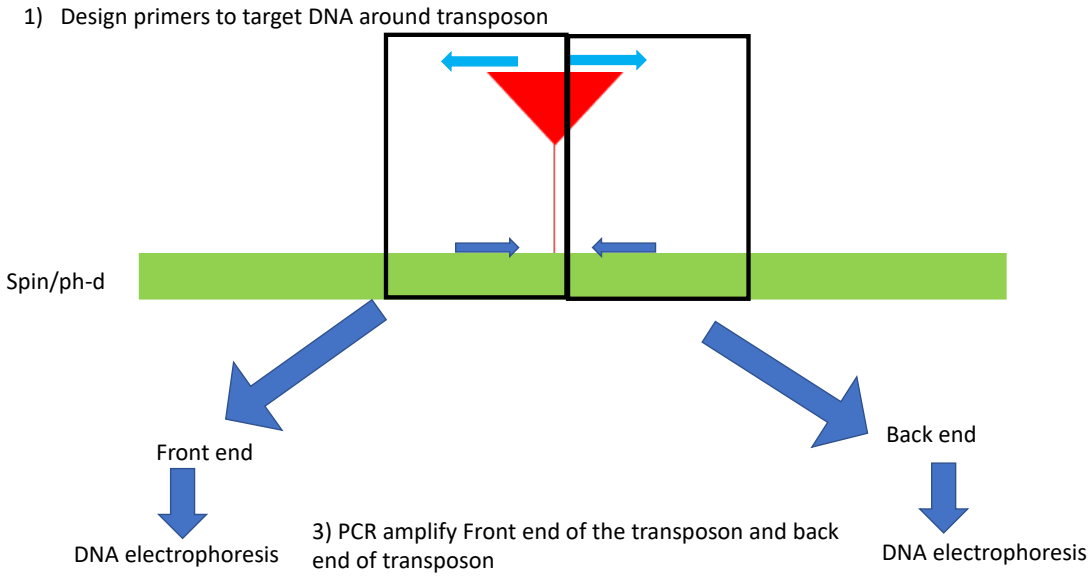


Figure 4: Genotyping protocol using PCR and DNA gel electrophoresis. The red upside-down triangle is how transposons are noted with a gene's DNA . For each transposon two primers were made to target the DNA upstream from the transposon (fwd or front end) and DNA downstream of the transposon (rev or back end). One primer was made for all of the P element transposons to target the inverted repeats of the transposon's DNA. For each transposon, two separate reactions were used to verify the reported location/determine if the transposon went through the seven generations of backcrossing. The first reaction was using the upstream primer plus the P element primer and the second reaction was using the downstream primer plus the P element primer.

Genotyping was done before backcrossing the *spin* P transposable element containing flies to verify the locations of these transposons were in the reported locations (flybase.org). Three *spin* transposable element containing flies were tested; EY10097, EY08566, K09905 (figure 5A). EY10097 PCR results did not have the anticipated band sizes with the front-end reaction results in primer dimers and the back-end reaction having band sizes over 100 bp too big. Sequencing revealed that the transposon was upstream of the reported position from Flybase (figure 5B). EY08566 band sizes were the intended sizes, verifying the transposon was in its correct position (figure 5C). The last transposon was K09905, and PCR revealed nonspecific banding in the front end of the reaction along with the predicted band size. Sequencing of the back-end reaction for this transposon revealed that the transposon was in the correct location (figure 5D). The non-specific banding seen (figure 5B) was from a fly that was originally balanced over the CyO chromosome. Those nonspecific bands were predicted to be the primers binding to DNA from the Cyo chromosome.

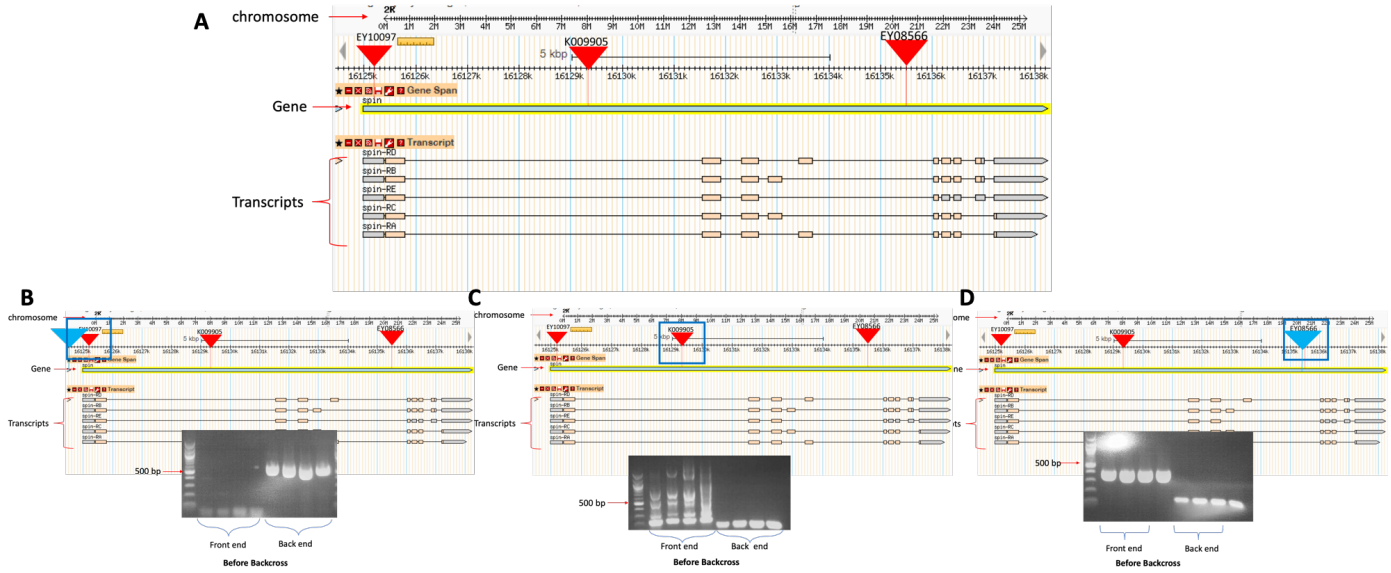


Figure 5: Genotyping to verify reported locations of transposons from Flybase. A) reported locations of transposons from flybase. B) EY10097 genotyping. Primer dimers from the front-end reaction and larger than expected bands from the backend reaction led me to believe that transposon was not in its reported position. These reactions were sequenced to verify that the transposon was not in reported position (red triangle) but upstream of the position by 300 bp (blue upside-down triangle. B) K00905 genotyping results before starting backcross. Nonspecific banding may represent the primers binding onto DNA from the extra CyO chromosome. Sequencing confirmed transposon was in reported position (red upside down triangle). C) EY08566 PCR before backcross, bands are expected sizes confirming the reported position (red upside-down triangle).

Backcross

The original stock flies containing the P-transposable elements in different regions of the *spin* gene did not have the same genetic background, meaning that testing for an ethanol sedation phenotype would not have resulted in data that was interpretable. As a result, these flies had to be backcrossed to wild-type flies. In order to determine if I was collecting flies that contained the transposon for each cross, it was important to use the mini-white marker within each gene to track the transposon throughout the backcross (figure 3). If the flies from each generation had red eyes, that meant they contained the mini-white marker within the transposon. By the end of seven generations each P-transposable element fly had the desired red eye, constituent with the transposons being tracked faithfully throughout the backcross

Genotyping after backcross

In order to verify that the transposon made it through the backcross, the same genotyping process was done for the fly DNA that came after the backcross. All three transposons had the similar PCR results from before the backcross, verifying that the transposon in each fly came out of the backcross (figure 6). In transposon K09905, the nonspecific bands seen in the front-end reaction from DNA before the backcrossed had gone away, suggesting that those bands were the result of the CyO chromosome (figure 6C). Once all three transposon locations were verified and came out of the backcross, I was able to test them for an ethanol sedation phenotype.

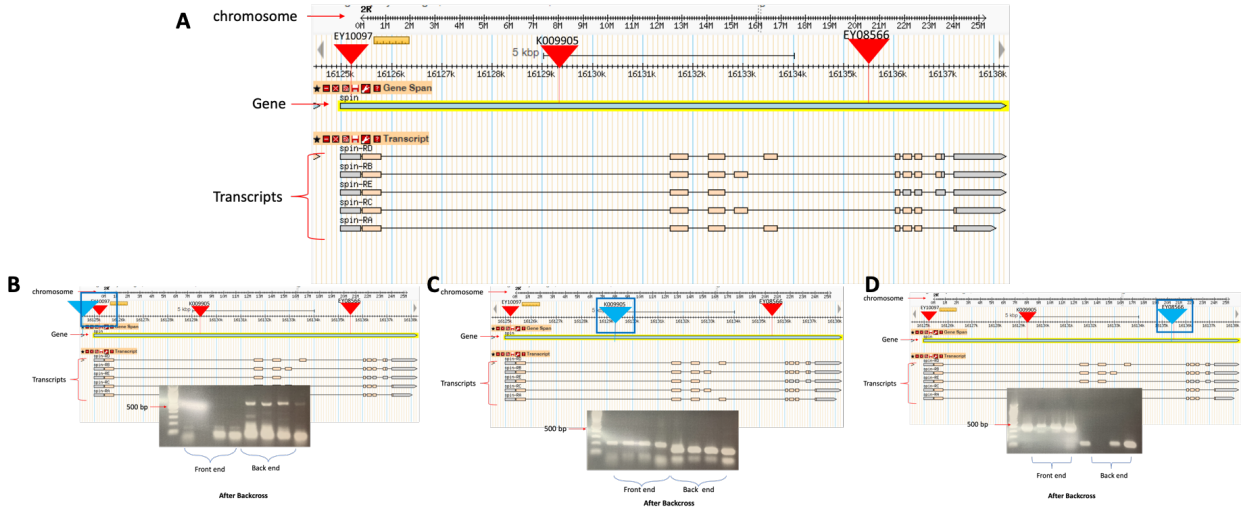


Figure 6: Genotyping with PCR with DNA from flies after completion of backcrossing. A) verified locations of all transposons of interest within the *spin* gene. B-D) verification that the transposon originally reported and verified location went through the backcross. Red upside-down triangle: original reported location of transposon from flybase. Blue upside down triangle: actual location of transposon D).

Ethanol sedation of spin P transposable element containing flies.

Ethanol sedation of each fly line containing the transposon was compared to control flies to determine if the transposon within the gene made the fly have an ethanol sedation phenotype. Out of the three P-element flies, two of them gave a statistically significant change in ethanol sedation (figure 7). This makes *spin* a strong candidate for influencing ethanol sedation, particularly when coupled with the *spin* RNAi data in figures 1 and 2.

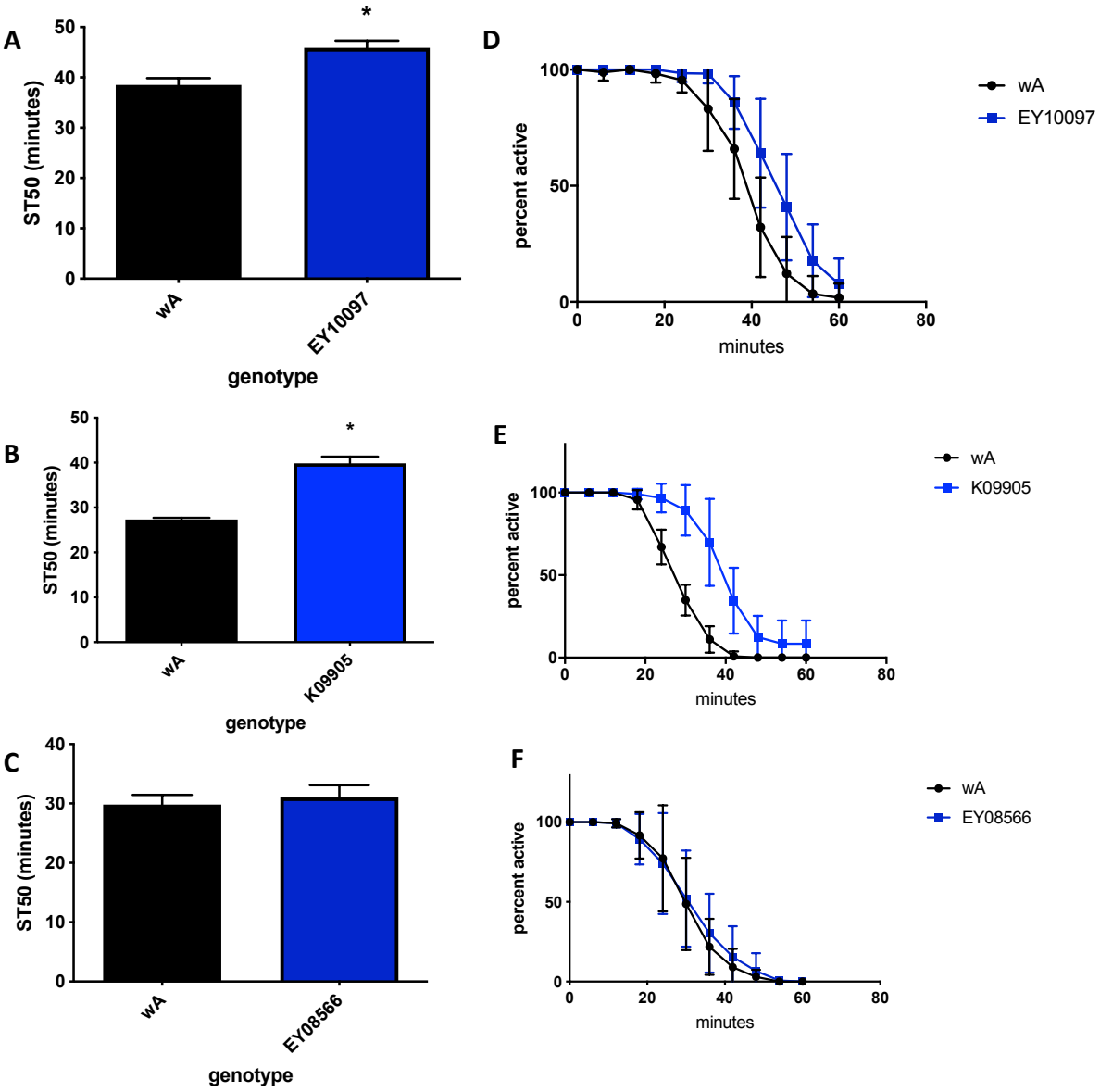


Figure 7: ethanol sedation of backcrossed flies containing *spin* P-transposable elements. A-C) ST50 calculated from panels D-F. D-F) Time course of ethanol sedation *unpaired t-test using Bonferroni's multiple comparisons $p<0.0001$ C) $p=0.0011$ D); n=12.

Discussion

Transposons are sequences of DNA inserted into a gene that can disrupt gene expression (Bellen et al., 2004). Three different P-element transposons that are inserted into the *spin* gene in different locations were backcrossed, genotyped, and tested in ethanol sedation. Genotyping was done to determine that the reported location of the transposon in Flybase was accurate and to determine that the transposon went through the seven generations of backcrossing. Two different transposon insertions in *spin* resulted in a significant change in ethanol sedation. These two transposons are candidate putative genetic manipulations of *spin* along with *spin* RNAi in ethanol sedation. Further testing of *spin* expression of these transposon insertion flies needs to be done to determine what the transposon is doing on the molecular level to determine how this genetic manipulation is affecting ethanol sedation.

C . spin as a candidate in ethanol sedation

Introduction

spin is a gene that is bound by Mef2 that showed a statistically significant change in ST50, making *spin* a candidate gene for ethanol sedation. *spin* (*spinster*) encodes a multipass transmembrane protein that is typically expressed in glial cells (Sweeney and Davis, 2002; Kim et al., 2017). This gene was first identified by its mutant phenotype which showed extreme mating refusal of females in response to male courtship (Sakurai et al., 2010). The spin protein is a late endosomal/lysosomal efflux permease for sugar carrying, and is also required in TGF-β signaling (Sweeney and Davis, 2002; Kim et al., 2017). Previous research has shown that this gene aids in cell death control (Sakurai et al., 2010) and helps control glial migration in the eyes during development (Yuva-Aydemir et al., 2011). Loss of function (LOF) *spin* mutants led to abnormal process of early endosome recycling and the accumulation of enlarged autophagosome/autolysosomes, which lead to abnormal head growth during embryonic stages of fly development (Kim et al., 2017). All these previous studies have shown that *spin* is important for development in the eye, head, and for major signaling pathways such as TGF-β, however no research has reported *spin* having influence on ethanol phenotypes in flies.

While *spin* and its mutants have shown phenotypes in mating behavior and development, no research has been done on *spin*'s influence on ethanol sedation. My previous ethanol sedation experiments demonstrated that neuronal expression of a *spin* RNAi showed a statistically significant change in ST50 compared to the controls. However, I need to determine that the expression of the RNAi in the neurons is the only factor contributing to that significant

ethanol sedation phenotype. In order to determine this a series experiments were done to verify that the RNAi expression was the only factor influencing ethanol sedation.

Results

Immunohistochemistry to verify knockdown of spin in neurons

Brains of flies expressing the *spin* RNAi in neurons were dissected and stained for spin (anti-spin) and neurons (anti-elav) using immunohistochemistry. Images were taken at 10x magnification to see if we were able to determine knockdown at the lowest magnification using confocal laser microscopy and image processing. Macroscopically, it appeared to show knockdown of *spin* compared to the control brains (figure 8A-C). However, formal image analysis showed no statistically significant change in *spin* expression (figure 8D).

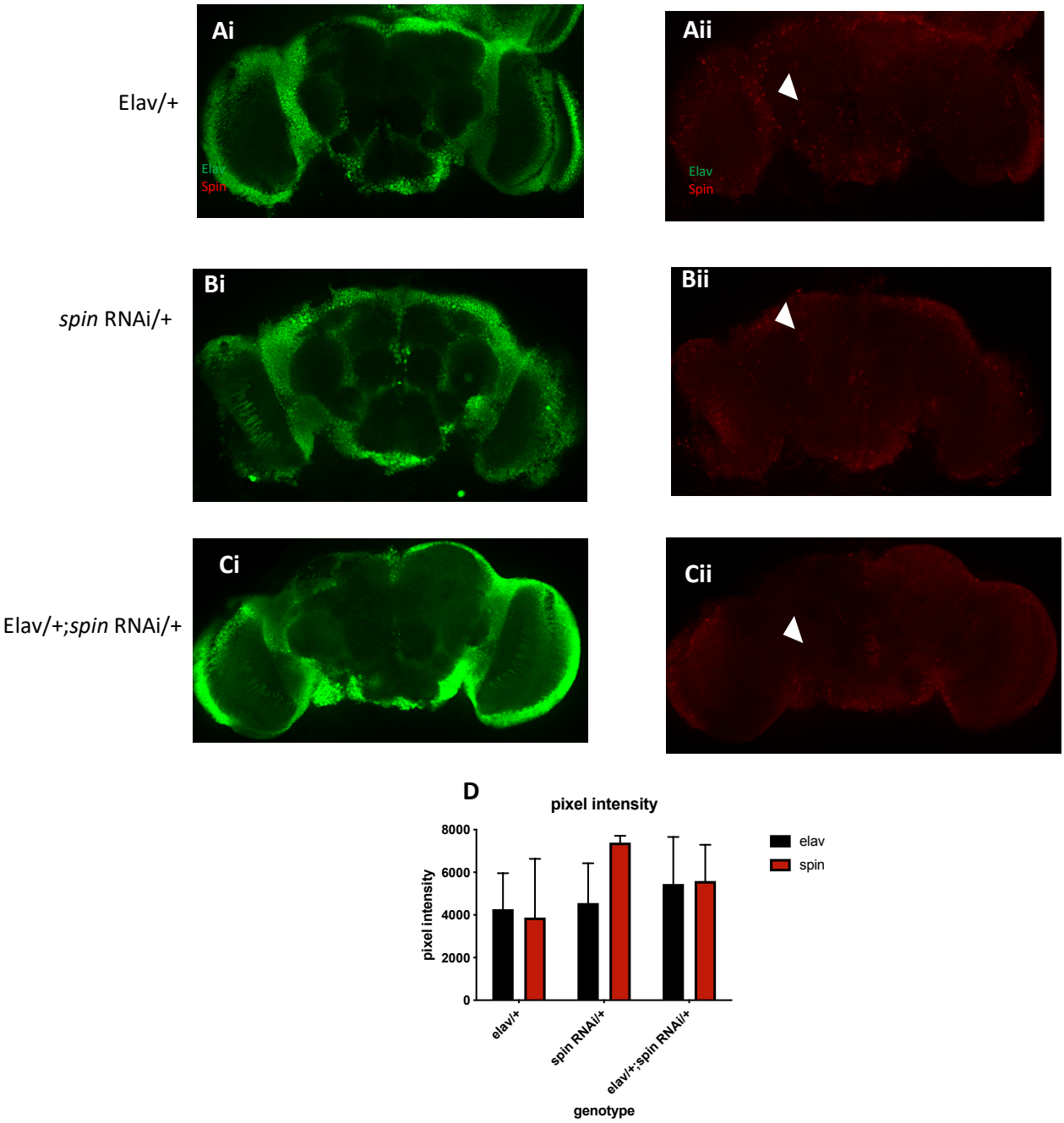


Figure 9: Whole brain spin expression and validation of *spin* RNAi transgene. A-C: representative (10x) confocal images of whole mount brains immunolabeled with anti-elav (neurons; green) and anti-*spin* (red). Macroscopically, in control flies (A, elav/+ and B, *spin* RNAi/+) expression of *spin* seems prominent (arrowheads) compared to neuronal knockdown (C). D) quantification of *spin* and neuron immunolabeling. Pixel intentsity derived from z-stack of all images of whole brains showed no statistically significant in expression compared to the control brains (Bonferroni Multiple comparisons (BMC); $p>0.9999$ for all genotypes; n=3). The quantification provided in 5D is derived from numerous images representing all portions of the 3 brains per genotype.

Discussion

spin is a Mef2-bound gene that is a candidate in ethanol sedation. Confocal laser microscopy and image analysis revealed no significant change in expression compared to controls, even though macroscopically it looks like there is a change (figure 8). Further imaging and analysis need to be completed to determine if the RNAi is knocking down its intended target of *spin* or off target genes. Further imaging of the co-localization of *spin* and neurons also needs to be evaluated since the literature suggests *spin* is primarily located in glial cells with some studies showing *spin* expression at the neuromuscular junctions (NMJs) (Sweeney and Davis, 2002; Yuva-Aydemir et al., 2011).

Chapter 4- Proposed analysis of *spin* and *Mef2* in ethanol sedation

A. Introduction

Previous results suggest that *spin* (*spinster*), a Mef2 bound gene, may influence ethanol sedation in *Drosophila melanogaster.* A gene screen using ethanol sedation of Mef2-bound genes showed that neuronal expression of an RNAi targeting *spin* showed a higher ST50, suggesting the flies are more resistant to ethanol. This led us to hypothesize that this gene may be an ethanol sedation candidate gene in flies. Further genetic manipulations of *spin* were done to further investigate *spin's* role in ethanol sedation. We used transposable elements inserted into the *spin* gene to determine if that also changed ethanol sedation. The transposons were backcrossed so they had the same genetic background and PCR experiments were done to determine the precise location of the transposons within the gene. The backcrossed *spin* transposon-containing flies were tested against control flies in ethanol sedation. Out of the three different transposons tested, two gave a statistically significant change in ethanol sedation (K09905 and EY10097). The third transposon tested (EY08566) showed no change in ethanol sedation suggesting that maybe the transposon insertion does not affect gene expression enough to give a change.

The genetic analyses I performed strongly suggests that *spin* influences ethanol sedation in flies. Several key questions that if answered would inform the validity of this possibility, however, remain unanswered. Here I describe a series of experiments to answer these questions to help determine if *spin* has a bona fide role in ethanol sedation in *Drosophila melanogaster.*

B. Is *spin* expressed in neurons and does the neuronal expression of *spin* RNAi or the transposon insertions affect *spin* gene expression?

Rationale

1. My major experimental hypothesis is that Mef2-regulated expression of genes in neurons is required for normal ethanol sedation. A fundamental prediction of this hypothesis is that *spin* (as a candidate Mef2-regulated gene) should be expressed in neurons. Additionally, determining the expression pattern of *spin* in the brain could support more refined hypotheses regarding specific brain regions, types of neurons, neurotransmitter systems, or possibly cellular compartments of neurons that might be important for the role of *spin* in ethanol sedation.
2. My studies show that expression of a *spin* RNAi transgene in neurons makes flies resistant to ethanol sedation, suggesting that *spin* functions within neurons and that the RNAi impacts ethanol sedation by knocking-down this expression. RNAi transgenes can have off-target effects (i.e. alter expression of other, unknown genes), making it essential to explicitly determine whether the *spin* RNAi in neurons is in fact knocking-down *spin* expression. My confocal imaging and image processing at 10x magnification showed no significant change in *spin* expression (figure 8D). However, this low-resolution analysis may not have revealed subtle changes in gene expression or changes in expression in a small subset of neurons. In order to further study these hypotheses, an approach would be to image and process at a higher magnification. Demonstrating the knockdown of *spin* in neurons using the *spin* RNAi will help link the gene to ethanol sedation and establish its function in neurons.

3. My experiments show that flies harboring either of two independent transposon insertions in or near *spin* have decreased ethanol sedation sensitivity. Based on the locations of the *spin* transposons and the ethanol sedation changes seen, it can be predicted that these insertions changed the expression of *spin.* The expression can decrease, increase, or stay the same. Determining the expression of *spin* in flies with the transposon insertions will help link *spin* to ethanol sedation and provide additional context for interpreting the results of *spin* expression studies in RNAi flies above. It is therefore critically important to look at the expression of *spin* in flies with transposons that impact ethanol sedation.

Hypotheses and/or predictions to be tested

1. *spin* is expressed in neurons.
2. The *spin* RNAi ought to show a statistically significant change in *spin* expression in the flies that have the neuronal expression of the *spin* RNAi compared to the controls when viewed at a higher magnification.
3. The transposon insertions in the *spin* gene should show a change in expression compared to the control flies since there was a change in ethanol sedation.

Approach

For 1, 2, and 3, immunohistochemistry, confocal laser microscopy (confocal imaging) and image processing will be used to measure gene expression of *spin*.

1. Flies of the following genotypes: *elav* control (neurons), RNAi control, and neuronal expression of *spin* RNAi will be dissected manually and fixed in paraformaldehyde (n=6 for each genotype). Flies will be stained for *spin* (anti-spin antibody) and neurons (anti-elav;

stains the elav protein, located in cell bodies). The brains of each genotype will be imaged using laser confocal microscopy and those images will be processed using image software in the VCU microscopy core to determine localization of the spin protein in relation to the neurons. This will be done at 10x and 40x magnification.

2. Flies of the following genotypes: *elav* control (neurons), *spin* RNAi control, and neuronal expression of *spin* RNAi will be dissected manually and fixed in paraformaldehyde (n=6 for each genotype). Flies will be stained for *spin* (anti-spin antibody) and neurons (anti-elav; stains the elav protein, located in cell bodies). Since there was no change in expression at 10x magnification, laser confocal microscopy will be done at a higher magnification (40x). Those images will be processed using image software in the VCU microscopy core to determine if there is a change in gene expression compared to genetic controls (elav and RNAi control).
3. Flies containing the transposon (EY10097 and K00905), and wild type flies (w[A]) will be manually dissected and fixed in paraformaldehyde (n=6 for each genotype) The brains will be stained for the *spin* protein (anti-spin antibody). These brains will be images using laser confocal microscopy and processed using image processing software in the VCU microscopy core.

Anticipated results

1. If we see that the spin protein has significant co-localization (overlapping) with elav (located in the cell body of the neuron), that will mean *spin* is expressed in neurons and most likely localized in the cell bodies of neurons. If we see that the spin protein has no

significant overlap with elav, then the spin protein may be located in another portion of neurons, subset of neurons, or in a non-neuronal cell type.

2. If we see a significant decrease in pixel/particle intensity in flies expressing *spin* RNAi compared to controls, then that will demonstrate that the RNAi is knocking down *spin* in the neurons. This will strongly support the possibility that the ethanol sedation phenotype seen in chapter 3 (figures 1 and 2) is the result of the *spin* knockdown. If we see no significant change in pixel/particle intensity compared to controls when the 40x magnification images are being processed, that will mean the *spin* RNAi is not detectably knocking down the *spin* gene.
3. If we see a significant change in pixel/particle intensity in flies with the transposons compared to controls, that will demonstrate the transposon insertion is changing *spin* expression. This would strongly support the possibility that the transposon insertion is affecting the ethanol sedation phenotype seen in chapter 3 (figure 8) via a change in *spin* expression. If we see no significant change in pixel intensity compared to controls when the images are being processed, that will mean the transposon insertion is not changing gene expression.

Potential pitfalls

1. Unable to determine spin location in neuronal cell bodies marked with anti-elav. If this happens I will stain the brains for spin and a new antibody targeting a different part of the neuron or other cell types. However, I will focus on neurons given the neuronal *spin* RNAi data in figure 8 from chapter 3.

2. There is no significant change in *spin* expression compared to the control genotypes at 40x magnification. I will measure co-localization between elav protein and spin protein in all control genotypes and neuronal RNAi expression. If there is a change in co-localization compared to the controls, it may suggest a functional change in *spin* expression due to RNAI or transposon insertions.
3. *spin* RNAi knocks down *spin* expression as expected, but *spin* expression is increased in one or more transposon lines. This result would suggest that either decreased or increased expression of *spin* in neurons could lead to blunted ethanol sedation sensitivity. This result would be consistent with a model in which *spin* is required for the normal function of specific neurons involved in ethanol sedation, and that decreased or increased expression of *spin* disrupts the function of those neurons, resulting in blunted ethanol sedation.

C. Does neuronal expression of *spin* RNAi or do transposon insertions in *spin* affect internal ethanol?

Rationale

It is possible that the primary impact of *spin* is to regulate how neurons respond to alcohol. Alternatively, it is possible that the primary impact of *spin* is on the uptake/metabolism of alcohol, and the altered uptake/metabolism of alcohol in flies with genetic manipulation of *spin* changes internal alcohol levels which has a secondary effect of altering ST50 values. These are two fundamentally different possible mechanisms for the role of *spin*, necessitating an explicit series of experiments to address them.

Hypotheses and/or predictions

The genetic manipulation of the *spin* gene in the flies will have one of two phenotypes:

1) Flies with genetic manipulation of the *spin* gene will have no significant change in internal ethanol suggesting the genetic manipulation has no effect on how the fly takes up or metabolizes the drug.
2) The genetic manipulation of *spin* will alter (presumably decrease) internal alcohol levels in flies.

Approach

We will measure the internal ethanol of flies with the genetic manipulation of neuronal expression of *spin* RNAi and transposon insertions in *spin* compared to control flies.

a. Neuronal expression of *spin* RNAi: These flies will be tested against two genetic controls, *elav* control (n=11 vials) and *spin* RNAi (n=11 vials)
b. Transposon insertion in *spin* gene: Flies with the *spin* transposon insertion (n=11) will be tested against wild type flies (w[A], n=11)

Internal ethanol will be tested at half ST50, and at ST50 for both a. and b. After exposure to ethanol for those two-time points flies will be homogenized. The fly's internal ethanol will be extracted with water and then measured using gas chromatography equipped with a flame ionization detector. The ethanol concentration will then be quantified by internal standard methods.

Anticipated results

If we see there is no change in internal ethanol in *spin* manipulated flies compared to control flies, that will suggest the genetic manipulation (neuronal expression of *spin* RNAi and transposon insertions) are not affecting the fly's ability to metabolize ethanol. This result would

indicate that the genetic manipulation of *spin*s in neurons (RNAi expression) or the transposon is affecting the change in ethanol sedation seen previously by altering how neurons respond to ethanol (i.e. having a pharmacodynamic effect).

Alternatively, if manipulation of *spin* changes internal ethanol, then that will suggest that the genetic manipulations to *spin* is influencing the uptake/metabolism of ethanol and the resulting altered internal ethanol concentration in the flies is affecting the change in ST50. This result would be consistent with the change in ST50 seen in flies with altered *spin* being secondary to the change in internal ethanol, raising the possibility that *spin* influences the uptake or metabolism of alcohol.

D. Is there a genetic interaction between *spin* and *Mef2*?

Rationale

Mef2 influences ethanol sedation and *spin* is a gene bound by Mef2*. spin* appears to also influence ethanol sedation. These findings support a model in which Mef2 is a transcriptional activator of *spin* and *Mef2* is therefore genetically upstream of *spin* for ethanol sedation. The goal of this section is to begin to formally address this model through an integrative molecular-genetic-behavioral strategy.

Hypotheses and/or predictions to be tested

Since (i) Mef2 binds *spin* and (ii) decreased expression of either *Mef2* or *spin* (presumed from my RNAi studies) causes ethanol sedation resistance, we postulate that *Mef2* is a positive regulator or *spin* expression. Consequently, we predict that decreased expression of *Mef2* should cause decreases expression of *spin*, and that increased expression of *spin* would be epistatic to decreases expression of *Mef2* for ethanol sedation. The studies described here will

address these two foundational predictions and will therefore aid our understanding of *Mef2, spin,* and the functional interactions between them in ethanol sedation.

Approach

Mef2 will be knocked down in the fly using RNAi. Those flies will be manually dissected and fixed in paraformaldehyde (n=6 for each genotype). Brains will be stained for Mef2 (anti-Mef2) and the spin protein (anti-spin). Fly brains will be images using confocal laser microscopy and image processing. Genotypes that will be tested are: *Mef2* RNAi knockdown fly, GAL4 control fly, and RNAi control fly (n=6 for each genotype). Once we can determine the expression pattern of *Mef2* and *spin* we will be able to study the molecular mechanism of *spin* and *Mef2* in ethanol sedation.

Ethanol sedation will be used to study the behavioral genetics of *Mef2* and *spin*. Four genotypes will be tested: GAL4 control, *Mef2* knockdown, overexpressed *spin* flies, and *Mef2* knockdown with overexpressed *spin* flies (n=8 for each genotype).

Anticipated results

Mef2 expression should decrease in flies expressing *Mef2* RNAi as the Grotewiel lab has previously demonstrated. If *spin* is likewise decreasing in the flies, the results will indicate that *Mef2* is an activator of *spin*. Since knockdown of *Mef2* and knockdown of *spin* both show resistance to ethanol sedation, overexpressing *spin* in *Mef2* knockdown flies will show a decrease in ethanol sedation resistance.

Potential pitfalls

If image processing of the brains shows no significant change in *spin* expression compared to the controls, I will reimage the brains at a higher magnification to determine the

expression of *spin* when *Mef2* is knocked down in the flies. If I see a significant change in *spin* expression at a higher magnification, then I will be able to conclude about the genetic interactions between *spin* and *Mef2*. If ethanol sedation results show a different pattern then described then those results will be inconclusive about the interactions between *Mef2* and *spin.*

E. Discussion

If we are able to see a certain pattern of results from these experiments, for all genetic manipulations to the *spin* gene, we will be able to determine if *spin* is a candidate gene in ethanol sedation in flies. If we see changes in *spin* expression we can conclude that the RNAi expression and the transposon insertion changes gene expression. If we also see no significant change in internal ethanol and locomotor activity compared to controls, then we can conclude that *spin* is a candidate in ethanol sedation in *Drosophila melanogaster.*

Chapter 5-Discussion

Mef2 is the *Drosophila melanogaster* ortholog to human *MEF2B*. Human *MEF2B* was found as gene that is related to SRE in a recent GWAS (Schmitt et al., 2019). When *Mef2* is knocked down using RNAi, there is a significant change in ethanol sedation. The flies with knockdown of *Mef2* show an increased tolerance to ethanol. Another study showed that *Mef2* binds 342 genes (Sivachenko et al., 2013), which leads us to believe that genes bound by *Mef2* may also play a role in ethanol sedation in *Drosophila melanogaster.*

A GSCAN was done on Mef2-bound genes to determine if any human ortholog genes are related to AUD. It was determined that 15 human ortholog genes out of the 342 *Drosophila* genes were associated to AUD. Using available RNAi reagents for the *Drosophila* genes, these genes were tested using ethanol sedation to determine if the RNAi knockdown of any of the genes influences ethanol sedation. Nine genes were tested using 36 RNAis and the *elav*-GAL4 driver under UAS expression. Only two genes showed significant change in ethanol sedation when using a single RNAi to knockdown the gene. *Spinster* (*spin*) and *Polyhomeotic-distal* (*ph-d*) RNAi showed significant change in ethanol sedation, showing an increase in ST50. Further research on both *spin* and *ph-d* revealed that *spin* would become the primary candidate gene of interest due to the availability of reagents.

The *spin* gene encodes a multipass transmembrane protein which is a late endosome/lysosome membrane protein with the amino acid sequence of lysosomal sugar carrier (Sweeney and Davis, 2002; Kim et al., 2017). In *Drosophila, spin* mutations were first identified for their effect on courtship behavior, which is extremely strong mate refusal by

females (Sakurai et al., 2010). Previous research demonstrated that loss of *spin* function also causes synaptic overgrowth at the neuromuscular junction (NMJ)(Sweeney and Davis, 2002). However, there has been no research into the effects of *spin* genetic manipulations on ethanol response in *Drosophila*.

Transposons are DNA segments that are inserted into a gene to affect gene function or expression (Bellen et al., 2011). These transposon insertion flies are made with different genetic markers, signifying that they are present in the genome. P transposable elements are one of the best studied DNA elements in eukaryotic DNA (Bellen et al., 2011). Using P transposable elements inserted into the *spin* gene can be another genetic manipulation to help determine if *spin* does influence ethanol sedation. Ethanol sedation of *spin* transposon insertion flies against control flies (wA) revealed a significant change in ethanol sedation. However, in order to verify that these genetic manipulations within *spin* do affect ethanol sedation in flies further tests need to be conducted.

Further testing of neuronal expression of *spin* RNAi flies and P transposon insertion *spin* mutant flies need to be conducted in order to determine if these manipulations are showing knockdown of the gene. Using immunohistochemistry and confocal imaging, the location of *spin* within neurons needs to be verified in order to help understand the molecular mechanism causing the ethanol sedation change seen. Also using these techniques, we will verify that *spin* RNAi is knocking down its intended target and not off target genes. We will also use immunohistochemistry and confocal imaging to determine the molecular mechanism of *spin* P element transposon insertions, since these insertions are known to disrupt gene function and expression.

Both the neuronal expression of *spin* RNAi and transposon insertions may disrupt gene expression or function, and may influence other behavior or metabolic pathways of the fly. In order to determine if these genetic manipulations influence non-ethanol related behavior or metabolic pathways of the flies two assays will be performed. In order to determine if the genetic manipulation affects metabolic pathways of the flies, internal ethanol of flies will be measured. If there is a significant change in internal ethanol compared to genetic control flies, then the ethanol sedation phenotype seen previously is the result of the genetic manipulation influencing the fly's ability to metabolize ethanol and not the fly's behavior response to ethanol.

By conducting the experiments described in chapter four on both genetic manipulations to the *spin* gene, we can further understand the mechanisms of *spin* that influence ethanol sedation in *Drosophila melanogaster.* It is known that the spin protein is located in late endosomal/lysosomal compartments in nerve and muscle tissue and is required for synaptic growth in cells (Sweeney and Davis, 2002). Mutations to *spin* have shown synaptic overgrowth at the neuromuscular junction (NMJ) which consequently led impaired neurotransmission at the NMJ. (Sweeney and Davis, 2002). Based on these observations, we can predict that knockdown of *spin* using RNAi and/or transposon insertions may lead to similar results. If that is the case, this could be the reason why there is a change in ethanol sedation using RNAi and transposon insertions.

The model organism, *Drosophila melanogaster,* is a powerful tool to study the genetic mechanisms of Alcohol Use Disorder (Singh and Heberlein, 2000). The behavioral responses to ethanol exposure in flies is very similar to that in humans, with effects of locomotor, sedation,

withdrawal symptoms, and tolerance (Grotewiel and Bettinger, 2015). With this in mind, it is important to use *Drosophila* as a way to study genes that known to be associated with AUD from association studies such as GWAS in humans. Once we can prove that the *Drosophila* ortholog genes influence alcohol response in flies, we can translate that back to the human level for further investigation.

By gathering genetic information influencing a fly's response to alcohol, it can give us a better understanding of the molecular mechanisms underlying AUD in humans. This can help lead to better treatment options for patients currently battling with this disease. Currently, most treatment plans for AUD involve 12-step groups, outpatient treatment by medical or nonmedical healthcare providers, and under prescribed medications (Kranzler and Soyka, 2018). However, by understanding the genetic factors influencing AUD researchers can start to develop gene targeted therapies to also aid in the treatment of Alcohol use disorder.

References

1. Adhikari P, Orozco D, Randhawa H, Wolf FW (2019) Mef2 induction of the immediate early gene Hr38/Nr4a is terminated by Sirt1 to promote ethanol tolerance. Genes, Brain Behav 18:1–9.]
2. Anon (2020) Alcohol Abuse.
3. Awofala AA, Jones S, Davies JA (2011) The heat shock protein 26 gene are required for ethanol tolerance in Drosophila. J Exp Neurosci 5:31–44.
4. Bellen HJ, Levis RW, He Y, Carlson JW, Evans-Holm M, Bae E, Kim J, Metaxakis A, Savakis C, Schulze KL, Hoskins RA, Spradling AC (2011) The Drosophila gene disruption project: Progress using transposons with distinctive site specificities. Genetics 188:731–743.
5. Bellen HJ, Levis RW, Liao G, He Y, Carlson JW, Tsang G, Evans-Holm M, Hiesinger PR, Schulze KL, Rubin GM, Hoskins RA, Spradling AC (2004) The BDGP gene disruption project: Single transposon insertions associated with 40% of Drosophila genes. Genetics 167:761–781.
6. Black BL, Olson EN (1998) Transcriptional Control of Muscle Development By Myocyte Enhancer Factor-2 (Mef2) Proteins. Annu Rev Cell Dev Biol 14:167–196.
7. Berger KH, Heberlein U, Moore MS (2004) Rapid and chronic: Two distinct forms of ethanol tolerance in Drosophila. Alcohol Clin Exp Res 28:1469–1480.
8. Black BL, Olson EN (1998) Transcriptional Control of Muscle Development by Myocyte Enhancer Factor-2 (Mef2) Proteins. Annu Rev Cell Dev Biol 14:167–196.
9. Bourne R, Bourne R (2010) ImageJ. Fundam Digit Med 9:185-188.
10. Brand NJ (1997) Myocyte enhancer factor 2 (MEF2). Int J Biochem Cell Biol 29:1467–1470.
11. Chan R (2013) VCU Scholars Compass Genetic Analysis of Ethanol Sensitivity and Tolerance in Drosophila.
12. Chan RF, Lewellyn L, Deloyht JM, Coffman S, Hewitt M, Bettinger JC, Ph D, Warrick JM, Ph D, Grotewiel M, Ph D (2015) NIH Public Access. 38:1582–1593.
13. Chen X, Gao B, Ponnusamy M, Lin Z, Liu J (2017) MEF2 signaling and human diseases. Oncotarget 8:112152–112165.
14. Cingolani P, Patel VM, Coon M, Nguyen T, Land SJ, Ruden DM, Lu X (2012) Using Drosophila melanogaster as a model for genotoxic chemical mutational studies with a new program, SnpSift. Front Genet 3:1–9.
15. Clarke TK, Adams MJ, Davies G, Howard DM, Hall LS, Padmanabhan S, Murray AD, Smith BH, Campbell A, Hayward C, Porteous DJ, Deary IJ, McIntosh AM (2017) Genome-wide association study of alcohol consumption and genetic overlap with other health-related traits in UK biobank (N=112117). Mol Psychiatry 22:1376–1384 Available at: http://dx.doi.org/10.1038/mp.2017.153.
16. Corl AB, Rodan AR, Heberlein U (2005) Insulin signaling in the nervous system regulates ethanol intoxication in Drosophila melanogaster. Nat Neurosci 8:18–19.
17. Craddock N, Owen MJ, O'Donovan MC (2006) The catechol-O-methyl transferase (COMT) gene as a candidate for psychiatric phenotypes: Evidence and lessons. Mol Psychiatry 11:446–458.

18. Crittenden JR, Skoulakis EMC, Goldstein ES, Davis RL (2018) Drosophila mef2 is essential for normal mushroom body and wing development. Biol Open 7.
19. Devineni A V., Heberlein U (2009) Preferential Ethanol Consumption in Drosophila Models Features of Addiction. Curr Biol 19:2126–2132 Available at: http://dx.doi.org/10.1016/j.cub.2009.10.070.
20. Ducci F, Goldman D (2008) Genetic approaches to addiction: Genes and alcohol. Addiction 103:1414–1428.
21. Duffy JB (2002) GAL4 system in Drosophila: A fly geneticist's Swiss army knife. Genesis 34:1–15.
22. Edenberg H, Foroud T (2013) Genetics and alocholism. Nat rev Gastroenterol hepatol 10:487–494.
23. Edwards AC, Deak JD, Gizer IR, Lai D, Chatzinakos C, Wilhelmsen KP, Lindsay J, Heron J, Hickman M, Webb BT, Bacanu SA, Foroud TM, Kendler KS, Dick DM, Schuckit MA (2018) Meta-Analysis of Genetic Influences on Initial Alcohol Sensitivity. Alcohol Clin Exp Res 42:2349–2359.
24. Engel GL, Taber K, Vinton E, Crocker AJ (2019) Studying alcohol use disorder using Drosophila melanogaster in the era of "Big Data." Behav Brain Funct 15:1–16 Available at: https://doi.org/10.1186/s12993-019-0159-x.
25. Flórez-Salamanca L, Secades-Villa R, Hasin DS, Cottler L, Wang S, Grant BF, Blanco C (2013) Probability and predictors of transition from abuse to dependence on alcohol, cannabis, and cocaine: Results from the national epidemiologic survey on alcohol and related conditions. Am J Drug Alcohol Abuse 39:168–179.
26. Garofalo RS (2002) Genetic analysis of insulin signaling in Drosophila. Trends Endocrinol Metab 13:156–162.
27. Grotewiel M, Bettinger JC (2015) Drosophila and Caenorhabditis elegans as Discovery Platforms for Genes Involved in Human Alcohol Use Disorder. Alcohol Clin Exp Res 39:1292–1311.
28. Haelterman NA, Jiang L, Li Y, Bayat V, Sandoval H, Ugur B, Tan KL, Zhang K, Bei D, Xiong B, Charng WL, Busby T, Jawaid A, David G, Jaiswal M, Venken KJT, Yamamoto S, Chen R, Bellen HJ (2014) Large-scale identification of chemically induced mutations in Drosophila melanogaster. Genome Res 24:1707–1718.
29. Hammer JH, Parent MC, Spiker DA, World Health Organization (2018) Global status report on alcohol and health 2018. Available at: http://www.who.int/substance_abuse/publications/global_alcohol_report/msbgsruprofiles.pdf%0Ahttp://www.ncbi.nlm.nih.gov/pubmed/29355346.
30. Heigwer F, Port F, Boutros M (2017) RNA interference (RNAi) screening in Drosophila (invited review, in prep.). Genetics 208:853–874 Available at: http://www.genetics.org/content/genetics/208/3/853.full.pdf.
31. Hodgson JW, Cheng NN, Sinclair DAR, Kyba M, Randsholt NB, Brock HW (1997) The polyhomeotic locus of Drosophila melanogaster is transcriptionally and post-transcriptionally regulated during embryogenesis. Mech Dev 66:69–81.
32. Johnson BA, Ait-Daoud N (1999) Medications to treat alcoholism. Alcohol Res Heal 23:99–105.

33. Juraeva D et al. (2015) XRCC5 as a risk gene for alcohol dependence: Evidence from a genome-wide gene-set-based analysis and follow-up studies in drosophila and humans. Neuropsychopharmacology 40:361–371.
34. Kempkes B, Robertson ES (2016) Epstein-Barr Virus Latency: current and future perspectives. Physiol Behav 176:139–148.
35. Kim JS, Park KS, Park O kyu, Kim SH, Jeon SH (2017) Novel phenotypes of Drosophila *spins*ter locus on the head formation during embryogenesis. Genes and Genomics 39:237–242.
36. Kranzler HR, Soyka M (2018) Diagnosis and pharmacotherapy of alcohol use disorder a review. JAMA - J Am Med Assoc 320:815–824.
37. Litten RZ, Ryan ML, Falk DE, Reilly M, Fertig JB, Koob GF (2015) Heterogeneity of alcohol use disorder: Understanding mechanisms to advance personalized treatment. Alcohol Clin Exp Res 39:579–584.
38. Liu M, Jiang Y, Wedow R, et al. Association studies of up to 1.2 million individuals yield new insights into the genetic etiology of tobacco and alcohol use. *Nat Genet*. 2019;51(2):237-244. doi:10.1038/s41588-018-0307-5
39. Matsushita S, Higuchi S (2014) Genetic differences in response to alcohol, 1st ed. Elsevier B.V. Available at: http://dx.doi.org/10.1016/B978-0-444-62619-6.00036-7.
40. mayo clinic (n.d.) Alcohol Use Disorder. July 2018.
41. Mulligan MK, Ponomarev I, Hitzemann RJ, Belknap JK, Tabakoff B, Harris RA, Crabbe JC, Blednov YA, Grahame NJ, Phillips TJ, Finn DA, Hoffman PL, Iyer VR, Koob GF, Bergeson SE (2006) Toward understanding the genetics of alcohol drinking through transcriptome meta-analysis. Proc Natl Acad Sci U S A 103:6368–6373.
42. Nicholson L, Singh GK, Osterwalder T, Roman GW, Davis RL, Keshishian H (2008) Spatial and temporal control of gene expression in drosophila using the inducible geneSwitch GAL4 system. I. Screen for larval nervous system drivers. Genetics 178:215–234.
43. NIH (n.d.) Alcohol Use Disorder.
44. Ojelade SA et al. (2015) Rsu1 regulates ethanol consumption in Drosophila and humans. Proc Natl Acad Sci U S A 112: E4085–E4093.
45. Osterwalder T, Yoon KS, White BH, Keshishian H (2001) A conditional tissue-specific transgene expression system using inducible GAL4. Proc Natl Acad Sci U S A 98:12596–12601.
46. Pon JR, Marra MA (2016) MEF2 transcription factors: Developmental regulators and emerging cancer genes. Oncotarget 7:2297–2312.
47. Potthoff MJ, Olson EN (2007) MEF2: A central regulator of diverse developmental programs. Development 134:4131–4140.
48. Reilly MT, Noronha A, Goldman D, Koob GF (2017) Genetic studies of alcohol dependence in the context of the addiction cycle. Neuropharmacology 122:3–21.
49. Robles-Murguia M, Hunt LC, Finkelstein D, Fan Y, Demontis F (2019) Tissue-specific alteration of gene expression and function by RU486 and the GeneSwitch system. npj Aging Mech Dis 5:2–6 Available at: http://dx.doi.org/10.1038/s41514-019-0036-8.
50. Robinson BG, Khurana S, Atkinson NS (2013) Drosophila larvae as a model to study physiological alcohol dependence. Commun Integr Biol 6:1–2.
51. Sakurai A, Nakano Y, Koganezawa M, Yamamoto D (2010) Phenotypic interactions of

*spin*ster with the genes encoding proteins for cell death control in Drosophila melanogaster. Arch Insect Biochem Physiol 73:119–127.
52. Sanchez-Roige S, Palmer AA, Fontanillas P, et al. Genome-Wide Association Study Meta-Analysis of the Alcohol Use Disorders Identification Test (AUDIT) in Two Population-Based Cohorts. *Am J Psychiatry*. 2019;176(2):107-118. doi:10.1176/appi.ajp.2018.18040369
53. Sandhu S, Kollah AP, Lewellyn L, Chan RF, Grotewiel M (2015) An inexpensive, scalable behavioral assay for measuring ethanol sedation sensitivity and rapid tolerance in Drosophila. J Vis Exp 2015:1–9.
54. Schmitt RE, Shell BC, Lee KM, Shelton KL, Mathies LD, Edwards AC, Grotewiel M (2019) Convergent Evidence from Humans and Drosophila melanogaster Implicates the Transcription Factor MEF2B/Mef2 in Alcohol Sensitivity. Alcohol Clin Exp Res 43:1872–1886.
55. Scholz H, Ramond J, Singh CM, Heberlein U (2000) Functional ethanol tolerance in Drosophila. Neuron 28:261–271.
56. Schuckit MA, Smith TL, Tipp JE (1997) The Self-Rating of the Effects of Alcohol (SRE) form as a retrospective measure of the risk for alcoholism. Addiction 92:979–988.
57. Schumann G et al. (2011) Genome-wide association and genetic functional studies identify autism susceptibility candidate 2 gene (AUTS2) in the regulation of alcohol consumption. Proc Natl Acad Sci U S A 108:7119–7124.
58. Setten RL, Rossi JJ, Han S Ping (2019) The current state and future directions of RNAi-based therapeutics. Nat Rev Drug Discov 18:421-446.
59. Singh CM, Heberlein U (2000) Genetic control of acute ethanol-induced behaviors in drosophila. Alcohol Clin Exp Res 24:1127–1136.
60. Sivachenko A, Li Y, Abruzzi KC, Rosbash M (2013) The transcription factor Mef2 links the drosophila core clock to Fas2, neuronal morphology, and circadian behavior. Neuron 79:281–292.
61. Southall TD, Elliott DA, Brand AH (2008) The GAL4 system: A versatile toolkit for gene expression in Drosophila. Cold Spring Harb Protoc 3:1–10.
62. Stebbins MJ, Urlinger S, Byrne G, Bello B, Hillen W, Yin JCP (2001) Tetracycline-inducible systems for Drosophila. Proc Natl Acad Sci U S A 98:10775–10780.
63. Sweeney ST, Davis GW (2002) Unrestricted synaptic growth in *spin*ster - A late endosomal protein implicated in TGF-β-mediated synaptic growth regulation. Neuron 36:403–416.
64. Wolf FW, Rodan AR, Tsai LTY, Heberlein U (2002) High-resolution analysis of ethanol-induced locomotor stimulation in Drosophila. J Neurosci 22:11035–11044.
65. Wu JS, Luo L (2006) A protocol for dissecting Drosophila melanogaster brains for live imaging or immunostaining. Nat Protoc 1:2110–2115.
66. Zhou H et al. (2020) Genome-wide meta-analysis of problematic alcohol use in 435,563 individuals yields insights into biology and relationships with other traits. Nat Neurosci.

Chapter 6-Appendices

A. GeneSwitch as an inducible gene expression system

1. *Introduction*

GeneSwitch (GS) is a GAL4 driver that is expressed in the presence of mifepristone (RU486) steroid (Osterwalder et al., 2001; Nicholson et al., 2008). The steroid can be administered in different ways; by either feeding the drug to the flies for an extended amount of time, or by immersing the animals in a steroid solution (Nicholson et al., 2008). The drug will bind to the GAL4 driver and change its conformation. The GAL4 driver is then in the correct conformation to bind to the UAS (upstream activation sequence) and turn on gene expression in a specific tissue (Nicholson et al., 2008) . The gene expression can be detected as early as three to five hours after administrating the drug to the flies, with maximal expression being observed 24-48 hours after administrating the drug (Osterwalder et al., 2001; Nicholson et al., 2008).

While this inducible expression system can be used to activate gene expression, it can also be used for expression of RNAi to knockdown gene function. GeneSwitch GAL4 has been used to express genes or RNAis in a number of different tissues including muscle, neurons, mushroom body-specific, eye-specific, glial cells, and tubulin (Nicholson et al., 2008). The goal of this project is to identify a neuronal GeneSwitch GAL4 driver that works using our standard RU486 feeding regime.

2. *Methods*

GeneSwitch Feedings

All GeneSwitch adult female flies were fed 1mM of mifepristone (RU486). 1mM of mifepristone was add to non-yeasted food vials as described in fly husbandry and stocks. All GS flies were fed the drug for a total of six days. On day three, all GS flies were flipped onto fresh non-yeasted vials with the mifepristone to prevent larval activity.

Locomotor activity

All Tetanus Toxin expressing GS flies were monitored on the drug for 6 days for decrease in locomotor activity. The number of flies that were dead/paralyzed were recorded.

Beta-Galactosidase Assay

All LacZ expressing flies were homogenized with drill and pestle. LacZ was extracted from flies using extraction buffer (1x PBS: 37 mM NaCl, 2.7 mM KCL, 8mM Na_2HPO_4, and 2mM KH_2PO_4. 1x Protease inhibitor (protease inhibitor cocktail, Sigma-Aldrich, St. Louis, MO.)) 100 uL of LacZ solution was added to 900 uL of 1mM Chlorophenol Red-β-D-galactopyranoside (CRPG) (Sigma-Aldrich, St. Louis, MO) and read in a spectrophotometer (ultrospec 2000, pharmacia Biotech, Piscataway, NJ.) at 562nm.

3. *Results*

Selecting GeneSwitch Drivers for screening

Six GeneSwitch lines were selected from the literature (Nicholson et al., 2008). These geneswitch lines were all previously screened in third instar larvae and adult heads. The six lines selected from this previous screen were all tissue specific to neurons (table 1). Two other lines

(stock #81013 and #43642) were selected from based on previous research from other collaborators.

stock	GS line	expression	LacZ induction	Tetanus toxin expression (28837 stock)	tetanus toxin expression (28838 stock)	source	citation
59949	P{w[+mW.hs]=Switch2}GSG3315-1, w[*]	ventral nerve cord cells, a few brain cells & chordotonal organs	no	no	no	BDSC	Nicholson et al., 2008, Genetics 178(1): 215--234
40333	y[1] w[*]; P{w[+mW.hs]=Switch2}betaTub56D[GSG5793]/CyO	ventral nerve cord, brain and sensory neurons	no	no	yes	BDSC	Nicholson et al., 2008, Genetics 178(1): 215--234
40286	w[*]; P{w[+mW.hs]=Switch2}GSG3763	ventral nerve cord and brain neurons.	no	no	no	BDSC	Nicholson et al., 2008, Genetics 178(1): 215--234
40283	w[*]; P{w[+mW.hs]=Switch2}GSG3630/TM6B, Tb[1]	ventral nerve cord, brain, sensory and neuromuscular junction neurons.	no	no	no	BDSC	Nicholson et al., 2008, Genetics 178(1): 215--234
40294	w[*]; P{w[+mW.hs]=Switch2}GSG4948	ventral nerve cord, brain, sensory and neuromuscular junction neurons	no	no	no	BDSC	Nicholson et al., 2008, Genetics 178(1): 215--234
43642	y[1] w[*]; P{w[+mC]=elav-Switch.O}GSG301	Neurons	no	lethal	lethal	BDSC	NA
81013	w[1118]; P{w[+mC]=MB-Switch}3/TM6C, Sb[1]	mushroom body expression.	no	no	yes	BDSC	NA
40251	P{w[+mW.hs]=Switch2}GSG5970, w[*]	subsets of ventral nerve cord, brain, sensory and neuromuscular junction neurons.	no	no	yes	BDSC	Nicholson et al., 2008, Genetics 178(1): 215--234

Table A. 1: Neuronal GeneSwitch lines tested with various reporter genes. All GeneSwitch lines tested were found in the literature and are expressed in neurons.

Tubulin GeneSwitch expression of Mef2 *RNAi in ethanol sedation*

Tubulin GeneSwitch is a GS line that is expressed in all tissues. Previous research has shown that Tubulin GS drives expression the reporter gene UAS-LacZ in a Beta-galactosidase assay (figure 1). Since this GeneSwitch driver has shown successful inducible expression, I used it to to express the *Mef2* RNAi previously reported in the lab and test in ethanol sedation. Tubulin induced knockdown in adult flies showed no significant change in ethanol sedation compared to the controls (figure 1). This result could be because the tubulin GS line is expressed in all tissues and that could be masking the effect of the *Mef2* knockdown. These results also give reason as to why a neuronal GeneSwitch driver is needed, an inducible knockdown of *Mef2* in a neuron-specific GeneSwitch driver could show different results.

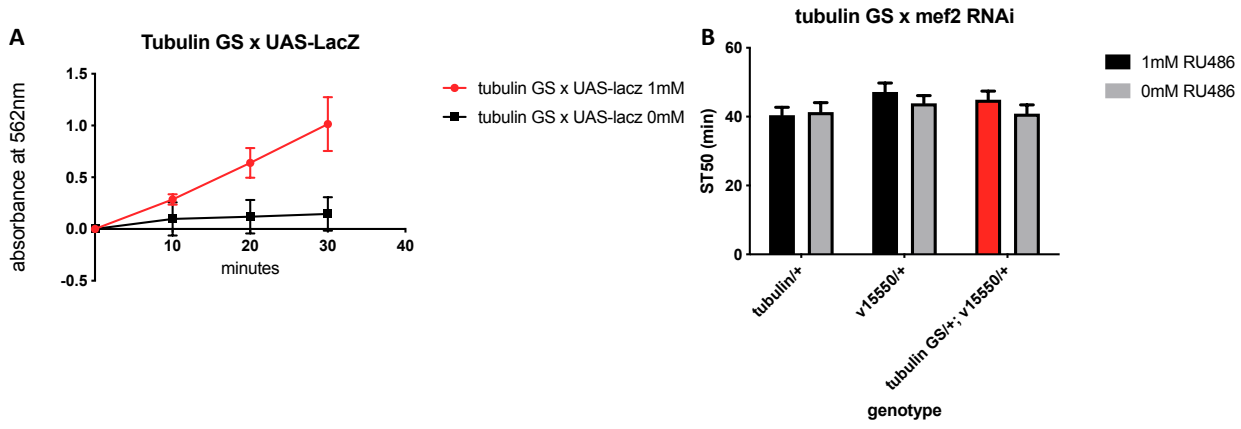


Figure A.1: Tubulin GeneSwitch used to induce gene expression. A) Tubulin GS used to express UAS-LacZ reporter gene. There was significant expression of lacZ compared to control flies. (Linear regression; $p<0.0001$; n=3) B) Tubulin GS used to knockdown *Mef2* by expressing *Mef2* RNAi transgene (#stock v15550). There was no statistically significant change in ethanol sedation (red) compared to controls. (2-way ANOVA; p=0.5744; Bonferroni's multiple comparisons BMC) $p>0.999$ for all genotypes; n=16).

LacZ and Tetanus toxin as a reporter gene to test GeneSwitch driver

UAS-LacZ is a reporter gene that was used to screen all 8 of the GS lines (table 1). LacZ expression was tested by measuring Beta-Galactosidase. No significant change was seen in the induced lines compared to the uninduced lines (figure 2).

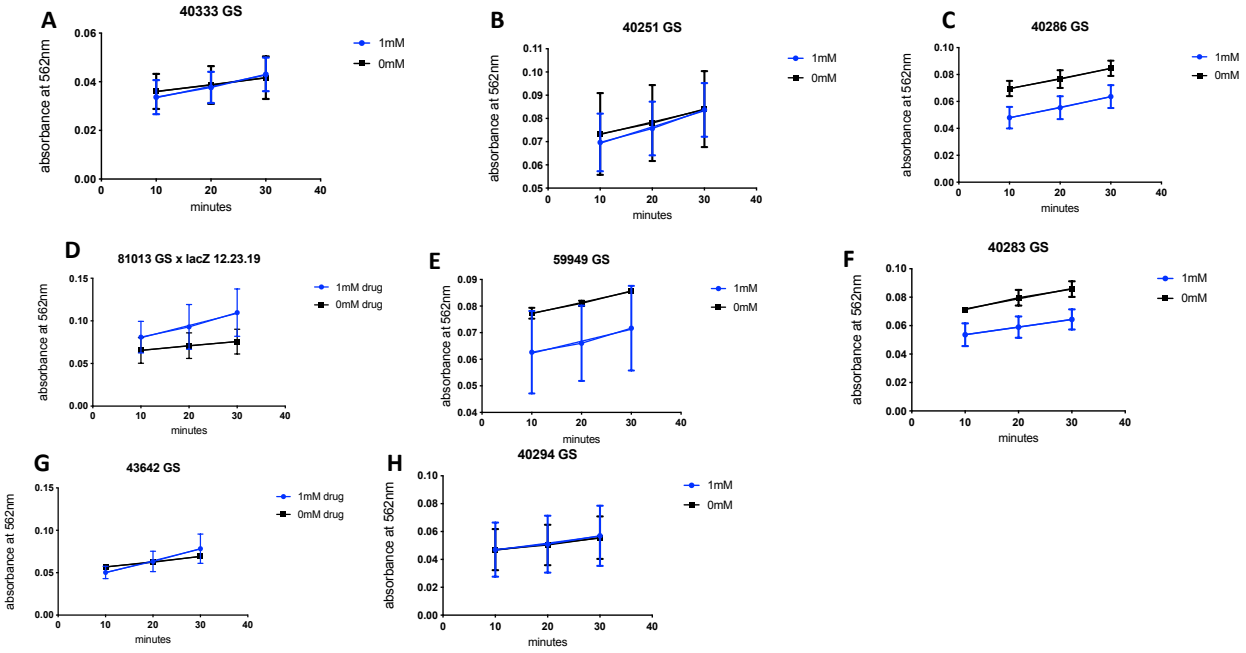


Figure A.2: Neuronal GS expression of UAS-LacZ reporter gene. A-H expression of LacZ in neurons. No significant change between flies that were exposed to the drug and those that were not being exposed. A-C) simple linear regression. A) p=0.6498, B) p=0.5915, C) p=0.9330, D) p=0.4123, E) p=0.9549, F) p=0.5915, G) p=0.1319, H) p=0.9456; n=3 for each genotype in all panels.

These lines were tested again using two different UAS-Tetanus toxin lines (stock #28837 #28838). When tetanus toxin is expressed, it blocks neurotransmitter release, this will paralyze and eventually kill the flies. Fly activity was measure over 6 days, recording the number of paralyzed/dead flies each day. There was no significant change in flies being fed the drug compared to the flies that did not get fed the drug (figure 3).

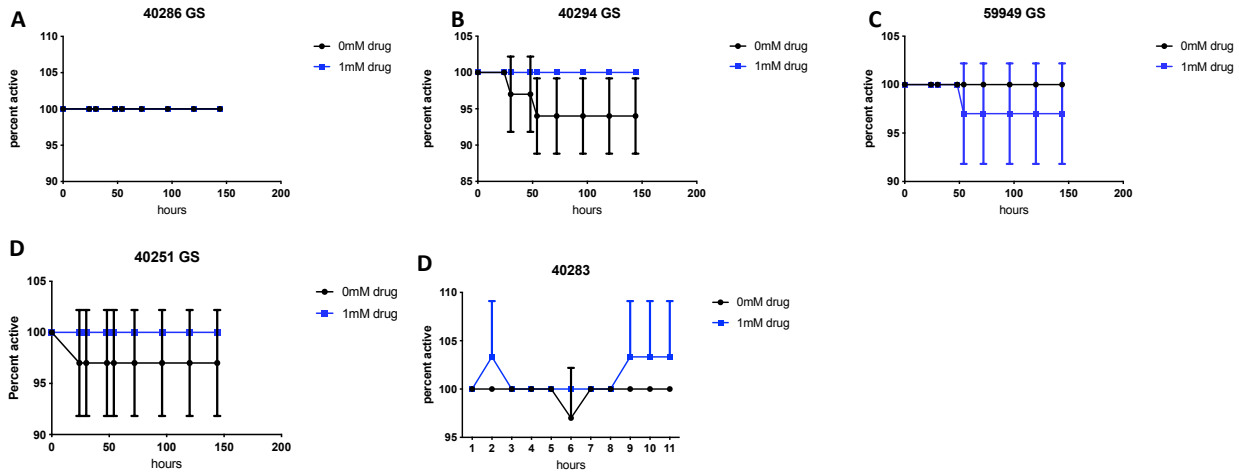


Figure A.3: Neuronal GS expression UAS-Tetanus toxin (#28837). A-D No significant change between flies that were exposed to the drug compared to those flies that were not. Simple linear regression B) p=0.0162, C) p=0.0753, D) p=0.5514; n=3 for each genotype in each panel.

The second tetanus toxin reporter gene (stock #28838) was then used to induce expression of the tetanus toxin. This tetanus toxin revealed stronger expression when induced with the neuronal GS driver (figure 4). Out of the eight lines tested for gene expression of the tetanus toxin, three showed significant change compared to the uninduced flies (table 1; figure 5). This suggests that UAS-tetanus toxin (stock #28838) was a successful transgene that showed gene expression in induced neuronal GS lines.

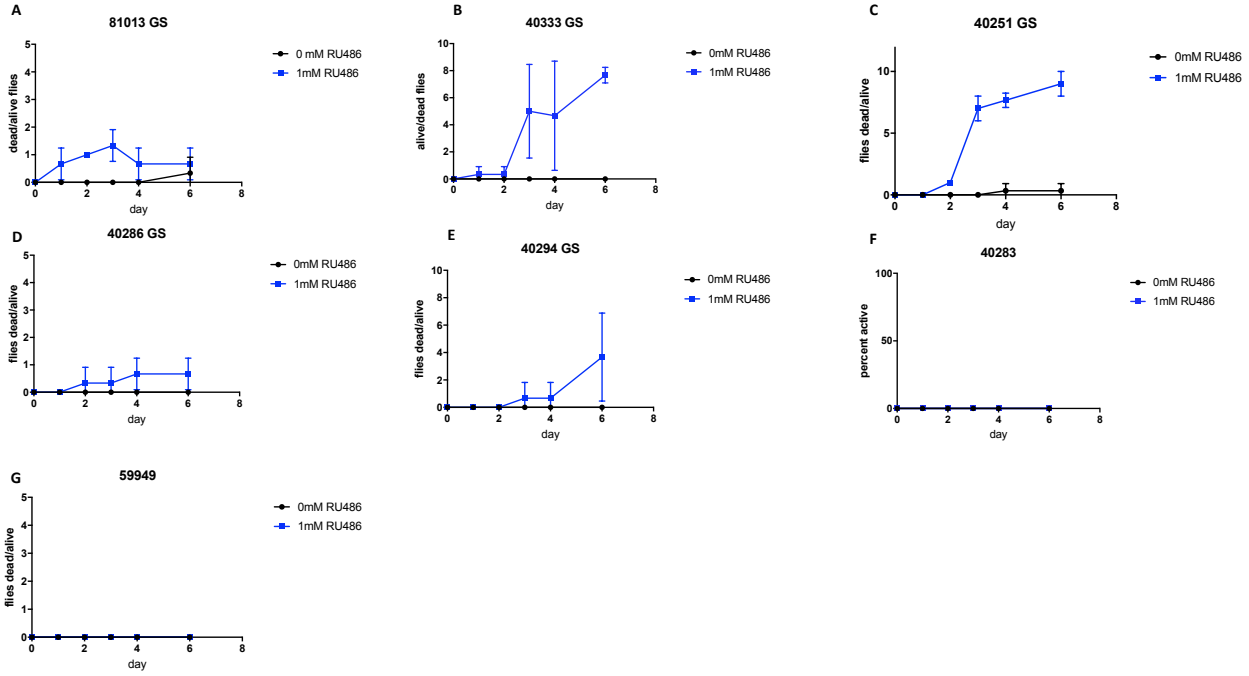


Figure A. 4: Neuronal GS lines expressing UAS-tetanus Toxin (#28838). A-E showed some change in expression compared to control flies. F-G no expression change. Simple linear regression, A) $p=0.6986$, B) $p<0.0001$, C) $p<0.0001$, D) $p=0.0157$, E) $p=0.0024$; n=3 for each genotype in each panel.

4. *Forward thinking*

Introduction

Eight neuronal GeneSwitch lines were selected from the literature and from colleagues (table 1). These Lines were tested to see if they activate a reporter gene (UAS-LacZ, UAS-Tetanus Toxin) using our standard feeding regime. Results showed no significant change in LacZ expression when flies were given the drug compared to those who did not get fed the drug (figure 2). I then used a different reporter gene, UAS-Tetanus toxin (stock 28837) to test these GS lines for gene expression. Once again, there was no change in expression compared to flies who were not fed the drug (figure 3). I tried a different UAS-Tetanus toxin (stock 28838) to test the lines and saw three GS lines that showed a significant change in expression (figure 4). These results gave us promising data to determine if there is a neuronal GS driver that will express a gene using our feeding regime.

Although we found three GS lines that showed expression of the UAS-Tetanus toxin (figure 4), there is still more testing that needs to be done to determine if these neuronal GS lines actually activate gene expression. We need to verify that the UAS-tetanus toxin (stock 28838) is a reporter gene that can test GS lines. We also need to verify that these GS lines do activate gene expression by using a different reporter gene (UAS-GFP). Once we can verify that these neuronal GS lines activate gene expression, these lines will be used to determine if neuronal knockdown of *Mef2* in adult flies has a change in ethanol sedation.

Rationale

Identify a neuronal GeneSwitch driver that will activate gene expression. Then use that neuronal GS driver to knockdown *Mef2* expression in adult flies to determine if that changes ethanol sedation compared to control flies.

Hypothesis

1) Identify a neuronal GeneSwitch driver that successfully activates gene expression of a reporter gene.
2) Once a neuronal GS driver has been properly screened, determine if there is a change in ethanol sedation when *Mef2* is knocked down in adults.

Approach

Since we have screened all eight neuronal GS drivers, and found three lines that gave promising results, the next steps are going to be further testing of these lines to determine if they are GS candidates.

1) The three GS lines that showed tetanus toxin expression will be crossed to UAS-tetanus toxin (stock 28838), given the food for six days, and monitoring the fly's activity over the six days they are exposed to the drug. Flies that are being fed the drug will be compared to flies that are not being exposed to the drug. This will be done to verify that the UAS-tetanus toxin is the right reporter gene to measure gene expression of these neuronal GS lines. Once this experiment is done, these GS lines will be tested again with a different reporter gene to confirm that the lines turn on gene expression when exposed to mifepristone. Neuronal GS flies will be crossed to UAS-GFP and fed the drug for six days. These flies will be compared to flies that are not fed the drug, and both sets of flies will have dissections of the brains. The brains will be imaged using confocal laser microscopy, those images will be

processed to determine if there is significant expression of GFP in flies fed the drug compared to flies not fed the drug.

2) Once a neuronal GS has been properly screened and identified, the GS driver will be used to knockdown *Mef2* in adult fly neurons and tested with ethanol sedation. These flies will be tested against the neuronal GS control and the *Mef2* RNAi control. All three genotypes will have flies that exposed to the drug and flies that are not exposed to the flies.

Anticipated results

1) If we see that at least one of the three GS drivers show significant expression of UAS-tetanus toxin and UAS-GFP, then that will confirm that those GS drivers do activate gene expression.
2) If we see that the neuronal knockdown of *Mef2* in adult flies has a significant change in ethanol sedation, then that will mean the knockdown of *Mef2* in adults also can impact the fly's response to ethanol.

Potential pitfalls

If we are unable to find a GeneSwitch driver that induces expression of a reporter gene then that can mean that the flies are not being properly exposed to the drug. In this case, we will change the feeding regime of the drug to the flies. If we find that the flies are being exposed to the drugs but they are not inducing gene expression then the next thing to do is to look for other neuronal GS drivers and re-test with the previously used reporter genes: UAS-Lacz, UAS-tetanus toxin, and UAS-GFP.

5. *Discussion*

GeneSwitch (GS) is a GALD4 driver that will express a gene or RNAi in the presence of mifepristone (RU486) (Osterwalder et al., 2001). We were interested in finding a GS driver that works in neurons because previous ethanol sedation experiments using Tubulin GS to knockdown *Mef2* showed no significant change in ethanol sedation (figure 12). Eight GS lines were tested using multiple reporter genes (UAS-LacZ, UAS-tetanus toxin). It was clear that measuring fly activity by expressing tetanus toxin was the best method to test these GeneSwitch lines. Further studies will need to be done verify that the three GS lines that showed expression of the tetanus toxin (figure 15) is consistent. From there, using the neuronal GS lines to knockdown *Mef2* in adult flies to see if there is a change in ethanol sedation will be done.

B. Protocols

1. *Basic Fly Handling and Husbandry*

A. Standard Fly Lab Lingo:

1. Stock or strain: a culture of flies with a particular genotype. Balanced stocks have a special chromosome called a balancer that is marked with a dominant phenotype and suppresses recombination on the corresponding sister chromosome. Balanced stocks are often weak (i.e. grow poorly).

2. Seeding: putting adult flies into a new bottle or vial. Also called 'setting-up'.

3. Transfer: moving flies without anesthesia from one vial or bottle to another. One-to-one transfer means moving flies from one bottle/vial to one new bottle/vial. Two-to-one transfer means moving flies from 2 vials/bottles to 1 new vial/bottle. Also called 'flipping'.

4. Clearing: removing all of the adults from a bottle or vial. Can be done with or without anesthesia.

5. Anesthesia: CO_2 used to temporarily immobilize flies.

6. Brood: refers to the number of times a set of adults has been used to seed bottles. Using flies for 2 broods is common, with 3 broods being possible in some cases.

7. white plus (w^+): indicates eye color. white minus (w^-) flies have white eyes. w^+ flies have eyes that can vary from light peach to deep red.

8. Food: All of our fly food currently has antibiotics on it (ampicillin, tetracycline and chloramphenicol; i.e. ATC). Yeasted (Y) food vials and bottles have live yeast on added. Yeasted food should be used for seeding new vials and bottles for *growing* flies. Non-yeasted (NY) food

has no yeast on it and should be used to *house* flies prior to behavioral studies and for *storing* virgin females and males prior to setting-up crosses.

B. Standard Fly Husbandry

1. Remove necessary number of yeasted bottles or vials from the cold room. Use bottles to grow lots of flies for behavioral or other large experiments. Use vials for smaller numbers of flies in limited scale crosses or other small-scale experiments.

2. Before putting in new flies, bottles and vials must be dried 2 hours to overnight in the environmental chamber so that all condensation on the walls evaporates. The food will pull away from the wall of the bottle or vial if they are over-dried. It is poor practice to use over-dried food.

3. Turn on the CO_2. Clean microscope, CO_2 pad and counter with ethanol. Clean before starting, between each genotype and after you are finished. Be sure the CO_2 is on before putting ethanol on the pad.

4. Open CO_2 to pipette, invert bottle or vial, insert pipette along cotton plug and tap bottle/vial gently. Flies will become anesthetized quickly and should fall onto the plug and/or the neck of the bottle/vial.

5. Click off CO_2 to pipette, remove CO_2 pipette from vial/bottle. Hold inverted bottle/vial over CO_2 pad. Remove plug and gently shake/tap flies onto pad into a pile. Return plug to bottle/vial and set aside.

6. Use brush or spatula to place anesthetized flies in a row and sort flies according to needs. Short CO_2 times are important. For collecting flies that will be used in behavioral studies, goals

are (1) all genotypes experience the same CO_2 exposure and (2) all flies are anesthetized for less than 5 min.

7. Set-up new bottles/vials by putting sorted flies from step 6 into dried bottles/vials. Anesthetized flies should be kept on the wall of the bottle/vial. If they fall into the food, many of them will stick there and die. Robust strains such as w[A] will do well with 10 females (♀, see below) per bottle or 3 females per vial. It is good practice to include a comparable number of males (♂, see below). Weaker stocks will need more females, up to as many as 50 per bottle and 15 per vial. When working with a stock that is new to you it is good practice to seed bottles or vials with a range of females (10-25/bottle for example) and then use an optimum number thereafter based on how the various bottles/vials grow.

8. Insert cotton plug, invert new bottle/vial and tap anesthetized flies onto the plug. Lay the bottle/vial on its side, label with genotype and date. First broods (i.e. bottles or vials in which the flies are new parents) are marked with a single slash.

9. Wait for flies to regain locomotor activity. Turn bottles/vials upright and place in environmental chamber to grow.

10. Beginning at around 4 days after seeding, check bottles/vials daily for larval activity (darkish band on top of food). When larval activity is obvious, either discard the adults or—if a second brood is needed—transfer adults to new bottles/vials (dried appropriately). Label second brood with genotype, date and two slashes.

11. Beginning at around 4 days after seeding the second brood, check bottles/vials daily for larval activity. Discard adults when larval activity is obvious. If necessary, a third brood is possible in some cases.

12. You should expect to see obvious larval activity 4 to 7 days after seeding and obvious pupae 5-10 days after seeding. New adults should begin emerging ~10 days after seeding. Some strains, especially balanced strains, can take up to 4 additional days to generate adults. Perfectly seeded bottle/vials will have robust larval activity followed by large numbers of pupae that populate the bottom three-fourths of the wall of the vial or bottle. Pupae will not typically be in the food or on the plug in these bottles. Large numbers of healthy adults suitable for experiments will emerge from perfectly seeded bottles/vials.

13. Common Problems: If your bottles/vials are too dry or wet (as described below), the resulting adults should not be used for behavioral, stress or gene expression studies. The resulting adults are fine genotype-wise and reproduction-wise, though, and can be used to set-up new bottles/vials as necessary.

a. Food too dry after 4-7 days of new adults in bottle/vial: The food should not be so dry that it detaches from the wall of the bottle of vial and the pupae are in the food. In cases like this, the food was either over-dried, there were not enough females placed in the bottle/vial, or possibly both. If this occurs across several strains that have grown well in the past, it is likely due to over-drying. If it occurs with a subset of strains, it is more likely due to insufficient numbers of females being used for those specific strains. The appropriate fixes are to decrease drying time, add more females next time, or both.

When you transfer flies from the first to second brood or when clearing the second brood, note the quality of the culture and food. If the food in some bottle/vials is detached from the wall after 7 days, go ahead and transfer/clear the adults and then add ddH_2O (NOT ETHANOL!) to the bottle/vial until the gap between the food and the wall is filled. In many cases this will

help the larvae quite a lot and you still might get a decent yield of adults, although they might be delayed a few days due to lack of water.

b. Food too wet after 4-7 days of new adults in bottle/vial: The food should not be so wet that it runs down the wall of the bottle/vial when it is inverted and the pupae are on the plug. If this happens, the food was not dried sufficiently before adults were added, too many adults were added, or possibly both. If this occurs across several strains that have not had this problem in the past, it is likely due to under-drying the food. If it occurs with only a subset of strains, it is more likely due to too many females being added in those specific strains. The fixes are to increase the drying time for bottles/vials, decrease the number of females used, or both.

If you notice that your bottles are too wet when transferring from the first to second brood or when clearing the second brood, you can put a folded Kim wipe in the bottle/vial so that it touches both the food and the plug. This will not result in a miraculous drying of the bottle/vial, but it can convert a bottle/vial that is far too wet into one that can be managed with some care.

C. The Basics of Setting-Up Crosses

1. You will need males (♂, mated or unmated) and virgin females (♀ with a 'v' on top) for your crosses. Grow bottles or vials as above for strains required to generate males and virgin females. For planning purposes, you can comfortably collect 50-100 males and/or 25-50 virgin females from a robust bottle. Likewise, you can probably count on collecting 15-20 males and 5-10 virgin females from each well-seeded vial.

2. Around day 10 after seeding, begin to collect virgin females, identified by their light body pigmentation and female genitalia (see below). Typically, one collects virgin females first thing in the morning, again around noon, and again last thing before leaving for the day.

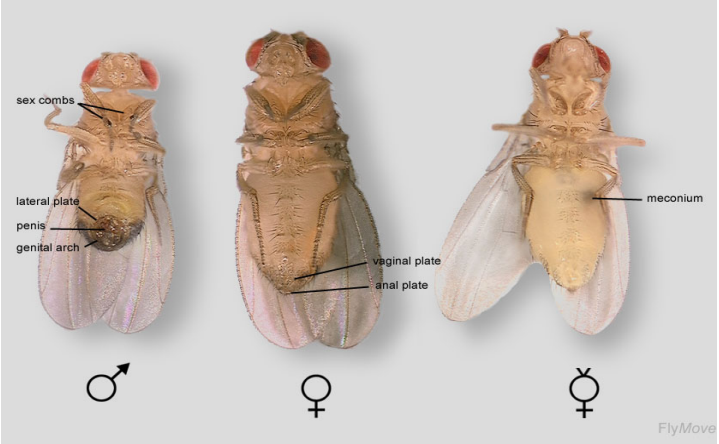
3. Keep virgin females in non-yeasted vials with no more than 25 females/vial. Label each vial with genotype, date and number of virgins collected. Keep collected virgins in environmental chamber until ready to use. One will often collect virgin females over several days until a sufficient number of virgin females has been collected. Also, it is convenient to store virgin females in upside-down vials.

4. When sufficient numbers of virgin females have been collected (~10% more than you plan to use) or when it is obvious that you will be able to collect all the virgin females you will need, collect all males into non-yeasted vials needed for your crosses. Males are identified by their male genitalia (see below).

5. Set-out yeasted bottles or vials to warm and dry as described above. On the day of the cross, check all virgin female vials for larvae using the microscope. Any vials with larvae MUST be discarded because at least one of the females has mated. Use only virgin females from vials with no larvae.

6. To set-up a cross, anesthetize the males and check them, anesthetize the virgin females on the same plate and check them, and put appropriate numbers of males and females into yeasted bottles/vials as described in steps B7-B9 above. Handle them thereafter as described in B10-B12 above.

7. Make sure that you know what progeny to expect from your crosses before you set them up.



2. Ethanol Sedation Assay

A. Day before assay

1. Collect flies (reared for behavioral assays) in groups of 11 (single sex) under brief CO_2 (~5 min) following standard procedures for behavioral assays. Collect only those flies that look healthy, are relatively the same size, have normal wings, and appear dry. Flies should be transferred from the CO_2 plate into an Eppendorf tube using a funnel and then dumped from the Eppendorf tube into a non-yeasted vial.

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

3. Dilute ethanol solution as necessary (85% is our standard concentration). ~250 ml of ethanol solution can be stored in a sealed 500ml bottle or other sealed container for a week without a problem. Make ethanol fresh weekly. Diluted ethanol is exothermic and should be stored overnight at room temperature before use.

B. Day of assay

1. For each vial of flies to be tested, you will need (a) a clean, empty food vial; i.e. testing vial, (b) a new Flug, (c) a silicone #4 plug and (d) 1.0 ml of ethanol solution (85% ethanol is our standard concentration).

2. Turn on humidifier and allow relative humidity in testing room to rise to 55-65%. Temperature should be 20-23°C. Record humidity and temperature on test log.

3. Have someone else in the lab assign a unique code to each group of vials for each genotype and—IMPORTANTLY—record the code for later. Place coded vials with flies in testing room to acclimate.

4. Label empty testing vials to match codes on fly vials from B.3.

5. Construct a testing log by entering the code for each vial into the Test Log E or Test Log EE sheet within the Excel Sedation file SA E EE 6 min SIGMOIDAL 2015.10.05. Use a random or cycling order. Add other pertinent information (% ethanol, sex, etc.) to the Test Log worksheet and print for use during testing.

6. Using the Test Log as a guide, arrange coded food vials with flies and empty testing vials into matching arrays with 4 vials in each row. The maximum possible number of vials that can be tested in a single experiment is 24 vials (i.e. 6 rows of 4 vials each).

7. Transfer flies from food vials into matched/labeled testing vials one at a time and immediately insert Flugs into testing vials until Flugs are a uniform distance below the vial tops. Use the Fluginator to push Flugs down into vials.

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

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

Start timer counting up at time 0 and immediately begin adding 1 ml of ethanol to the Flug in the vials for the first row/set of 4 vials. Add ethanol to the vials at 5 second intervals in the order they will be tested. Add ethanol to the Flugs in a circular motion so that all ethanol is absorbed as uniformly as possible. When ethanol has been added to all four testing vials in the set, insert a silicone #4 plug in each vial to seal it.

At times 1, 2, 3, 4 and 5 min on the timer, add 1 ml of ethanol to the second, third, fourth and fifth sets of 4 vials, respectively. Continue inserting #4 plugs after adding ethanol to each set of 4 vials.

10. At time 6 min, test the first set of 4 vials by gra*sping* the first vial with thumb and forefinger and then tapping gently on the table three times to knock flies to the bottom of the vial. Tap the other 3 vials in the set the same way at 5 second intervals. 30 seconds after tapping the first vial, count and record the total number of flies that are sedated. Count and record the number of sedated flies in the other 3 vials at 5 second intervals. Flies are scored as sedated if they do not appear to have productive locomotion. The specific schedule is:

Vial	Tap	Assess
1	6 min 0 s	6 min 30 s
2	6 min 5 s	6 min 35 s
3	6 min 10 s	6 min 40 s
4	6 min 15 s	6 min 45 s

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

11. At time 12 min, test the first set of 4 vials again as described in B10 and continue testing the second, third, fourth, fifth and sixth sets of vials at 13, 14, 15, 16 and 17 min, respectively.

Continue testing flies as described in B10 and B11 until all flies are sedated (typically 60-90 min).

12. Record the total number of flies in each vial.

13. Clean-up is (a) turn off humidifier, (b) remove #4 plugs for washing and reuse, (c) discard Flugs/vials/flies, (d) remove any trash from and straighten up testing room and (e) turn off light in testing room.

14. Enter the total number of flies in each vial and the number of flies sedated at each time point in the Test Log within the Excel worksheet. Percent Active flies will be automatically calculated and graphed below the Test Log. Press 'Ctrl + s' to calculate ST50s for each vial and sort the data by group in the Sorted Data worksheet.

15. Note any flagged data in Sorted Data worksheet. Consider excluding data that looks qualitatively poor.

M Grotewiel, R Schmitt, K Lee: 7/2014, 3/2015, 7/2016

3. Fixation, Staining, Mounting, Imaging and Analysis Protocol for Whole Adult *Drosophila* Brains

Dissection: Day 1

1. Anesthetize flies and place adults of the appropriate age, genotype and gender into a three-well dish on ice

2. Fill another three-well dish with PBT and place it under the dissecting microscope

3. Place a 0.5mL snap cap tube containing 500µL of freshly prepared 4% paraformaldehyde on ice

4. With gentle, sharp forceps, remove the brain from the head cuticle in the PBT solution. Place it into the 4% paraformaldehyde on ice

- Sharpen forceps with sharpening stone prior to dissection
- Use clean forceps during dissection – have wet Kim wipes ready to wipe fly parts onto
- The best way to access the brain = dunk flies into PBT belly up. Use second forcep to remove the proboscis, which will leave a large hole in the flies' head. Grab ahold to opposite sides of the hole with both forceps, and slowly pull the forceps away from each other to remove the cuticle.
    - Demonstrated here: https://www.youtube.com/watch?v=j4rVa7JCzdg&t=5s
- Things to remember:
    - The retinas and eye pigment auto-fluoresce. Make sure they are fully removed!
    - Too much connective tissue left on the brains (white stringy stuff) will cause the brains to float, which makes them harder to stain. Remove as much as possible

5. Repeat for the remaining flies. Place up to 10 brains in a tube

Staining:

*** Wear black gloves. Always check pipet tips to ensure brains were not sucked up with solution. All excess solution (unless noted otherwise) can be discarded into a small beaker and washed down the sink ***

*** Make sure all primary and secondary antibodies have been optimized for concentration. Directions on how to do this are in the Notes section below***

Day 1 (~3 hours)

1. Place the 0.5mL tube containing brains in 4% paraformaldehyde onto a nutator. Allow the brains to fix for 20 min at room temperature

2. Remove the tube from the nutator and place it into a tube rack at room temperature. Allow the brains to settle to the bottom of the tube. Use a P-200 pipet to remove the paraformaldehyde. Dispose appropriately under fume hood.

3. Add 0.5mL PBT to the tube. Close and invert the tube. Allow the brains to settle to the bottom. Remove the PBT. Repeat once more (=2 quick washes at room temp)

4. Add 0.5mL PBT to the tube. Place on nutator to wash for 20 min. Repeat two more times (=3 20min washes at room temp)

5. Remove the PBT from the brains and add 0.5mL block solution (5% NGS). Place brains on nutator to block for (at least) 30 min at room temp.

- A little bit longer than 30 min is okay

6. Remove block solution from the brains. Add the primary antibody solution at the pre-determined concentration. Place on nutator at 4°C for 2 nights (approx. 36-48 hours).

** can be increased to 1 week if antibody penetration isn't good

Day 3 (~1.5 hours)

7. Remove primary antibody and store it at 4°C. The antibody can be reused roughly three + more times.

- Always record the dates and number of times used!
- These antibodies could have bacteria in it ☹ beware

8. Add 0.5mL PBT to the tube. Close and invert the tube. Allow the brains to settle to the bottom. Remove the PBT. Repeat once more (=2 quick washes at room temp)

9. Add 0.5mL PBT to the tube. Place on nutator to wash for 20 min. Repeat two more times (=3 20min washes at room temp)

10. Remove PBT. Add secondary antibody at the pre-determined concentration. Place on nutator at 4°C for 2 nights (approximately 36-48 hours).

** can be increased to 1 week if secondary antibody penetration isn't good

(this should not be an issue with AlexaFlours – if troubleshooting penetration issues with an Alexa secondary antibody, start with increasing the primary antibody exposure time)

Day 5 (~2.5 hours)

11. Remove the secondary antibody and discard

12. Add 0.5mL PBT to the tube. Close and invert the tube. Allow the brains to settle to the bottom. Remove the PBT. Repeat once more (=2 quick washes at room temp)

13. Add 0.5mL PBT to the tube. Place on nutator to wash for 20 min. Repeat two more times (=3 20min washes at room temp)

** DAPI can be added to one of the 20 min washes to stain nuclei

14. Remove PBT and add 200µL SlowFade. Allow brains to settle in SlowFade at 4°C. This normally takes 1 hour

** Do not leave brains in SlowFade for more than 24 hours

Mount brains:

1. Label slide with pencil

2. Use a P-200 pipet tip to transfer the brains in SlowFade from the tube onto a mounting slide. Only transfer a little SlowFade at a time (100uL or under) - avoid adding excess SlowFade. Excess SlowFade can be removed with a kimwipe.

3. Using forceps, carefully align the brains for ease of imaging.
   - Place flat against the bottom of the slide. Arrange in a line
   - No great videos for this, but you'll get the idea through these two videos…
     - https://www.youtube.com/watch?v=ga9wre91T7M
     - https://www.youtube.com/watch?v=yHoN1PzsTaA (first four min only)

4. Arrange two broken no. 1 coverslips on the microscope slide on opposing sides of the mounted brains in SlowFade (aka bridge). Do not let the coverslips touch the SlowFade. Gently place a no. 1 coverslip on top of the bridge to cover the brains
   - This technique prevents the brains from becoming too compressed under the top coverslip

5. Slowly pipet SlowFade under the coverslip on the bridge (surface tension will allow the SlowFade to go under the coverslip). Continue administering the SlowFade until the whole coverslip is close to full.
   - Avoid adding too much SlowFade that it starts going under the broken coverslips – this will cause the "bridge" to float, which makes sealing the edges (next step) difficult.

6. Seal the edges of the coverslip with nail polish. Start by making brush strokes that are perpendicular to the coverslip and bridges. Let dry. Repeat with brush strokes that are parallel to the coverslip and bridges.

7. Store at 4°C in a dark slide holder

** mounted slides are good for several months at 4°C and several years (3+) at -20 or -80°C

8. Image using confocal microscope

Imaging:
*** try to image as quickly after mounting onto the slide as possible. Within the same week is usually best***

1. Get training on the Confocal LSM700 prior to imaging brains alone
    - This can be arranged through the microscopy core

2. Key points for clean, publishable images:
    - Always use Nyquist sampling. This is easy on Zen software – make sure the "Optimize" button on the Acquisition panel is pressed

    - Always ensure that the lens pinhole is 1 airy unit wide. This can be achieved by pressing the 1AU button on the Channels panel. If the lens is changed, the 1AU button must be pressed again

    - Image with the highest number of Averaging possible (on the Acquisition panel). For single slices, aim for 8 or 16. For Z-Stacks, aim for 4 or 8.

    - Beware of photo-bleaching! This occurs when a sample is exposed to the laser for a long period of time. It results in the sample not be as excitable (i.e. bright fluorescence) as it was before.
        - Z-Stacks 35 min and under are typically fine. Less averaging = quicker Z-stack. Wider slice thickness = quicker Z-stack. If playing around with the settings for one brain, make sure all brains within a sample are imaged using the same settings. You will need to report these come publication time!

    - When optimizing a sample, choose the brain within the sample/slide that looks best (most intact, all optic lobes attached, no obvious forcep marks). Optimize this brain using the Range Indicator tool (little blue background, little red within tissue i.e. the background (blue) and saturation (red) are controlled for). Image all brains within the sample (i.e. on that slide and maybe other slides) using these microscope settings.

Image Analysis:
The Microscopy core can help with this! Computers with image analysis software are free to use and the programs are always updated

<u>Determining overall pixel intensity with ImageJ</u>
i.e. how fluorescent an image is – this is a good analysis if trying to determine how much an RNAi knocked down protein expression

1. Open image in Image J

2. Image Adjust Brightness/Contrast Set chose the range (specific to the BIT depth used while imaging)
    - This information is in the raw data file on Zen. But should always be 16 BIT

3. IF two channels (colors, flours) used while imaging, but you only want to analyze one channel…
   - Image color split channels

4. Select image you want to analyze by clicking Analyze histogram (if Z-Stack hit analyze all) record the mean and the standard deviation

5. Repeat with remaining images!

Determining Manders co-localization with ImageJ
i.e. how much two proteins overlap – this is a good analysis if trying to determine how much of a protein is in a cell type

1. Open image in Image J

2. Image Adjust Brightness/Contrast Set chose the range (specific to the BIT depth used while imaging)
   - This information is in the raw data file on Zen. But should always be 16 BIT

3. Split the two-color channels: Image color split channels

4. Method one: analyze colocalization lots of options here! I would typically do coloc 2 and Manders overlap coefficient reported, can interpret as a percent

5. Method two: process image calculator multiple the images together the inverse of the result shows colocalization

Notes:

How to make the solutions noted above:

0.3% (vol/vol) PBT solution:
Add 1.5 Triton-X 100 to 498.5mL 1 X PBS. Store at room temperature

4% (vol/vol) paraformaldehyde:
In a 0.5mL snap cap tube, add 100µL 20% w/v paraformaldehyde to 400µL PBT. Prepare fresh and place on ice.

5% (vol/vol) normal goat serum (NGS): Add 50µL normal goat serum to 950µL PBT. Store this block solution for short periods at 4°C (24-hour max)

Primary antibody: Dilute the primary antibody in freshly prepared 5% NGS. A 0.5mL tube requires 400µL of diluted antibody. Diluted primary antibodies can be reused up to 3 times. Store in 4°C for up to 1 month-ish

Secondary antibody: Dilute the secondary antibody in freshly prepared 5% NGS. A 0.5mL tube required 400µL of diluted antibody. Prepare fresh and discard after use.

DO NOT do any staining steps (addition of primary antibody, secondary antibody, SlowFade) at a shorter time at room temperature in Sanger Hall.

- The air in Sanger Hall has too much bacteria and the bacteria grow too fast at room temperature and will take over the brains if the 4C steps are removed.

Bacteria growth under the confocal microscope transmittable light will look stringy and/or black. It will compromise the exterior regions of the brain at first, but as time goes on the whole brain will be compromised. Slides with brains with bacteria cannot be re-imaged weeks/months after the slide is made.

What does antibody penetration issues look like??

If the fluorescent signal is strong on the exterior portions of the brain, but not on the interior portions of the brain. Each antibody is different, so if playing around with antibody concentrations does not fix this (see below), choose a concentration that looked good on the exterior portions of the brain and increase the amount of time the brains are exposed to this concentration at 4C

So, you got a new primary or secondary antibody – how do you optimize concentrations so it is ready to use??

Step One:

1. Check the literature – have any fly labs used this antibody for whole mounts before (adults or larva) – what concentration did they use??
2. Dissect brains (w[A] or similar control stock) and stain with multiple concentrations chosen based on previous literature (i.e. 1 concentration per tube of brains)
    - Example: If previous literature used a 1:200 concentration, you can try…
    - 1:100, 1:200, 1:500, 1:1000, 1:2000
    - For this optimization, it is okay to only have 2-3 brains per concentration tube!
3. Image and decide which concentration produces the best image in your hands. What other labs have used may not be the best concentration for you!

Step Two:

4. Dissect brains (w[A] or similar control stock)
5. Stain with new reagent only!
    - So, if a primary antibody, only stain with the primary antibody and not a secondary antibody. If a secondary antibody, only stain with the secondary antibody and not a primary antibody.
6. Image – Are you able to detect any fluorescent signal at reasonable power/gain settings?
    - This will determine whether that reagent and the concentration you have chosen produced any fluorescence on its own
    - If no fluorescence is detectable – good! You are ready to use this concentration for experiments!

- If fluorescence is detectable – repeat with a lower concentration.
    - If that doesn't work… Talk to the microscopy core people about controlling for background noise. It will be an extra step in your experimental image analysis, but the data will still be trustworthy!

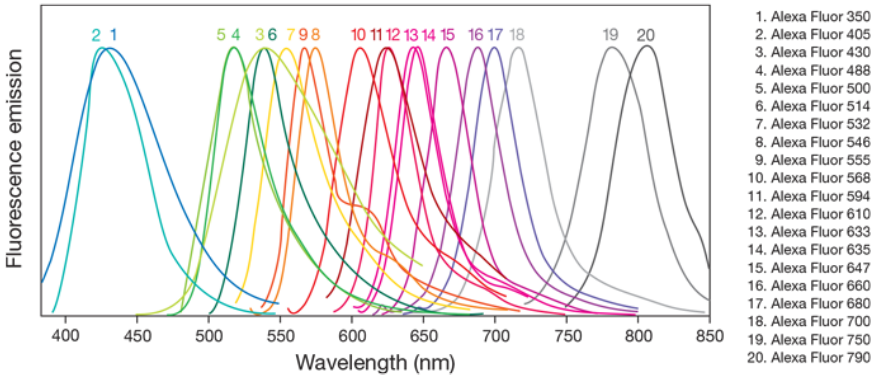
Selecting antibodies for co-localization experiments:

1. Make sure the antibodies for the two different proteins you want to label are from different hosts
    - Example: Mouse anti-X, Rabbit anti-Y

2. Make sure the secondary antibodies are specific for their intended proteins primary antibody, and are also from different hosts
    - Example: Goat anti-mouse, Chicken anti-rabbit

    What will not work:
    - Primary: Mouse anti-X, Rabbit anti-Y
      Secondary: Goat anti-mouse, Mouse anti-rabbit
      the Goat anti-mouse secondary will recognize the mouse anti-X primary antibody and the mouse anti-rabbit secondary antibody

3. Make sure the fluorescent probes excitation/emission wavelengths do not overlap/minimally overlap
    - What works well:
        - Alexa488 and Alexa568 (minimally overlapped)
        - Alexa488 and Alexa647 (not overlapped)

    - Use this chart as a guide if purchasing new secondary antibodies for co-localization experiments:
    - 

    - UAS-GFP, UAS-RFP, UAS-YFP, etc. can be used as alternatives to antibodies. However, there excitation/emission wavelengths should be treated the same as antibodies if doing co-localization

KML updated 5/2019-staining protocol adapted from Luo, 2006. *A protocol for dissecting* Drosophila melanogaster *brains for live imagine or immunostaining.*

4. Basic Polymerase Chain Reaction (PCR) Protocol

1. Dilute primers to 10 pmol/µl. The primers in the Primer Boxes in the -20°C freezer are at 1 nmol/µl (1000 pmol/µl), so those need to be diluted 1:100 in dd H2O.

2. Thaw in hand and/or store on ice:

primers

Taq Buffer (in Taq box; includes $MgCl_2$)

2.5 mM dNTPs

dd H_2O

3. Each PCR sample or tube should contain:

1-5 µl template (e.g. 200 ng purified gDNA or 2-5 µl of squish prep)

5 µl forward primer

5 µl reverse primer

5 µl Taq Buffer

5 µl 2.5 mM dNTPs

0.3 µl Taq polymerase

X µl dd H_2O to 50 µl total

Making each individual sample separately is cumbersome, so make a Master Mix for n+1 samples (i.e. the number of samples you need to amplify plus 1). The master mix contains all components that will be used in multiple samples. For example, if amplifying several different gDNAs with the same primer pairs, the Master Mix should contain everything except the gDNAs. When making the Master mix, add all components except for Taq polymerase, vortex to mix, add the Taq polymerase, then vortex to mix.

4. Aliquot the Master Mix into labeled 0.5 ml thin-walled PCR tubes and add remaining component(s).

5. Close lids and pulse vortex. If necessary, gently tap tubes to bring liquid to bottom.

6. Place tubes in thermocycler, adjust thumb-wheel on lid until you start to feel a SMALL amount of pressure, set program to appropriate program, enable heated lid, press Proceed. A run takes ~3 hours, but can vary depending on cycling parameters.

5. DNA sequencing Protocol (GeneWiz)

1. PCR amplify the region to sequence

2. Run a gel on your PCR product using 3 μL with ladder (using 10 μL)

3. Add 2μL exosap to PCR product (~7μL) and run exosap cycle

4. Using the gel results, determine the relative concentration of your bands (2x brightness, 3x brightness, etc.) and compare it to the ladder legend to determine concentration of your DNA (legend should be on the ladder box or attached to the fume hood-note the μL the legend is for).

5. Using the included table for GeneWiz use Premixed purified PCR product samples, dilute your DNA with ddH_2O to the specified concentration

6. pipette 10μL of your diluted template per PCR tube (note: label tubes on the side with your initials and the number of tube (IN1, IN2, IN3) and label the top with the number of the tube

7. add 5μL of your diluted primer (5μM) to each reaction tube (total volume is 15μL)

8. Enter your information into GeneWiz and print off the mailing sheet. To ship, seal the top of your tubes with parafilm and seal in a bag. The mailing box is on floor 4 (MMRB)

DNA Type	DNA Length (include vector)	Template Concentration in 10 µl	Template Total Mass	Your Primer Total Picomoles	Premixed Volume* (Template + Your Primer)
Plasmids	<6 kb	~50 ng / µl	~500 ng	25 pmol	15 µl
	6 - 10 kb	~80 ng / µl	~800 ng		
	> 10 kb	~100 ng / µl	~1000 ng		
Purified PCR Products	<500 bp	~1 ng / µl	~10 ng	25 pmol	15 µl
	500 - 1000 bp	~2 ng / µl	~20 ng		
	1000 - 2000 bp	~4 ng / µl	~40 ng		
	2000 - 4000 bp	~6 ng / µl	~60 ng		
	> 4000 bp	Treat as plasmid	Treat as plasmid		

Example:

I run my gel using 10 µL of a 100bp New England BioLabs Ladder. I use 3µL of my PCR product in each well. On the gel, my bands appear approximately 3.5x brighter than the brightest band on the ladder. The brightest band on the ladder according to the legend is 95ng (in 10µL, as specified by the legend). So, 95 ng x 3.5 is 332.5ng in my sample (which is 3µL). this means my concentration of DNA in 1 µL of my sample is 110.8ng (332.5/3 gives us ng/1µL). I then run my remaining 7µL of PCR product with 2µL exosap for the exosap cycle. When that (9µL) reaction is finished, I must dilute my sample to the desired concentration (for 788 bop of purified PCR product, the end product must be 2ng/µL). Since I have 775.7 ng DNA in my 9µL sample (110.8 ng x 7µL PCR product +2µL exosap), I must add ddH_2O to get a total volume of 9uL product + exosap, I must add 378.8uL ddH_2O to get a final concentration of 2 ng/µL. I should then take 10µL of this final product and add 5uL of my diluted (1:20) sequencing primer, then send my order in for sequencing.

Vita

Katlyn Marie Myers was born on May 30th, 1996 in Gretna, Louisiana and is an American citizen. She attended Longwood University (Farmville, VA) from 2014 to 2018 and received her Bachelor of Science in Biology. She came to Virginia Commonwealth University in 2018 in the Molecular Biology and Genetics program. She joined Dr. Mike Grotewiel's laboratory, which is in the Department of Human and Molecular Genetics.