DEFINING GENES AND CIRCUITS AFFECTING NAÏVE AND EXPERIENCE-DEPENDENT ALCOHOL PREFERENCE IN DROSOPHILA MELANOGASTER

APPROVED BY SUPERVISORY COMMITTEE:

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Adrian Rothenfluh, PhD

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Helmut Kramer, PhD

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David Self, PhD

_____
Jonathan Terman, PhD

DEDICATION

To my family, friends, professors, and those who in my nostalgia induce a smile on my face.

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By

Shamsideen A. Ojelade

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Shamsideen A. Ojelade

The University of Texas Southwestern Medical Center at Dallas

Graduate Advisor: Adrian Rothenfluh, PhD

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ABSTRACT OF DISSERTATION

Despite alcohol being one of the most used and abused drugs in the world, the molecular mechanisms underlying alcohol abuse disorders remain largely unknown. In this dissertation, I utilized the model system *Drosophila melanogaster* to identify genes and circuits affecting ethanol-induced behaviors. From an unbiased genetic screen, I identified a novel gene that affects ethanol consumption in both flies and humans. Ras suppressor 1 (Rsu1) is required in the adult *Drosophila* nervous system for normal sensitivity to ethanol-induced sedation, and acts upstream of Rac1 and downstream of integrin to regulate the actin cytoskeleton. In a two bottle choice assay called the capillary feeding (Café) assay, loss of Rsu1 causes immediate heightened alcohol preference compared to wild type's initial naïve aversion. In contrast, flies specifically lacking Rsu1 in the mushroom bodies show normal initial aversion to alcohol, but then fail to acquire ethanol preference like normal flies do. Our data show that not only is Rsu1 required for normal alcohol responses, it suggests that different anatomical brain structures in flies control distinct alcohol behavioral responses. In humans, we find that polymorphisms in RSU1 are associated with brain activation in the ventral striatum during reward anticipation in adolescents and alcohol consumption in both adolescents and adults. Together, these data suggest a conserved role for integrin/Rsu1/Rac1/actin signaling in modulating reward-related phenotypes, including ethanol consumption in flies and humans.

Using a modified Café paradigm, we investigated whether dopamine plays a role in both the aversive and experience-dependent properties of alcohol. I show that distinct subsets of DA neurons innervating the Fan-shaped body (FSB) and Mushroom body (MB) mediate naïve alcohol aversion (NAA) and experience-dependent alcohol preference (EDAP) respectively in flies. Furthermore, Rac1-dependent actin alteration in these anatomical structures (FSB and MB) also mirror dopaminergic-induced neuronal activity in these circuits suggesting that dopamine functions upstream of Rac1-signaling to affect alcohol preference in flies. Taken together, my dissertation suggests a conserved role for dopamine and the integrin/Rsu1/Rac1/Cofilin/Actin signaling pathway in modulating drug-induced behavioral plasticity across phyla, and highlights *Drosophila* as an effective model for integrative translational research.

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List of Abbreviations

AC	Adenylate Cyclase
ADH	Alcohol Dehydrogenase
Arf6	ADP-Ribosylation Factor 6
Arfaptin	Ribosylation Factor-Interacting Protein
Arfip	Arfaptin
aru	arouser
AUTS2	Autism Susceptibility Candidate 2
ANOVA	Analysis of Variance
AUD	Alcohol Use Disorder
BACE1	Beta-Site Amyloid Precursor Protein Cleaving Enzyme 1
BDNF	Brain-Derived Neurotrophic Factor
BK	Big Potassium Channel
CA	Constitutive Active
CAFE	Capillary Feeder
CAM	Cell Adhesion Molecule
CAMKII	Calcium/Calmodulin Dependent Kinase
cAMP	Cyclic Adenosine Monophosphate
CDC42	Cell division control protein 42 homolog
CDE	Clathrin-Dependent Endocytosis
CGN	Cerebellar Granule Neurons
CNS	Central Nervous System
CPP	Conditioned Place Preference
Ctl	Control
dAlk	*Drosophila* Anaplastic Lymphoma Kinase
Lmo	LIM-Domain Only
DA	Dopamine
DAn	Dopaminergic neurons
DN	Dominant Negative
DopR	Dopamine Receptor
Dop1r1/ D1R	Dopamine D1 Receptor 1
Dop2r/D2R	Dopamine D2 Receptor
ECM	Extracellular Matrix
EB	Ellipsoid Body
EDAP	Experience-Dependent Alcohol Preference
EEA1	Early Endosome Antigen 1
ELAV	Embryonic Lethal Abnormal Vision
EGFR	Epidermal Growth Factor Receptor
ERK	Extracellular Signal-Regulated Kinase
EPS8	Epidermal Growth Factor Receptor Kinase Substrate 8
F-actin	Filamentous Actin

Fas II	Fasciculin II
FITC	Fluorescein isothiocyanate
F-MRI	Functional-Magnetic Resonance Imaging
FSB	Fan-Shaped Body
FOXO	Forkhead Box Protein O
GABA	Gamma-Aminobutyric Acid
GABAR	GABA Receptor
G-actin	Globular actin
Gal80^{ts}	Gal80-Temperature Sensitive
GAP	GTPase Activating Proteins
GDP	Guanine Diphosphate
GEF	Guanine Nucleotide Exchange Factor
GFP	Green Fluorescent Protein
GTP	Guanine Triphosphate
JPK	Jasplakinolide
ics	*icarus*
IGF	Insulin Growth Factor
InR	Insulin Receptor
KCs	Kenyon Cells
Km	Michaelis Constant
Kra	Krasavietz
Lat. A	Latrunculin A
LIMK-1	Lin-11, Isl-1 and Mec-3 Kinase 1
LOR	Loss-Of-Righting
LTP	Long-term Potentiation
L-LTP	Late-Long-term Potentiation
LTD	Long-Term Depression
LTM	Long-Term Memory
MAPK	Mitogen-Activated Protein Kinase
MB	Mushroom Body
MBONs	Mushroom Body Output Neurons
MID	Monetary Incentive Task
MTOR	Mammalian target of Rapamycin
mys	Myospheroid (integrin mutation in Beta receptor subunit)
NAA	Naïve Alcohol Aversion
NFBC	North Finish Birth Cohort
NMDA	N-Methyl-D-Aspartic Acid
NMJ	Neuromuscular Junction
NPY	Neuropeptide Y
NPF	Neuropeptide F
PAL	Paired Anterior Lateral
PAM	Paired Anterior Medial
Par-1	Polarity Kinase-1

PI	Preference Index
PI	Pars Intercerebralis
PI3K	Phosphoinositide 3-Kinase
PINCH	Particularly Interesting Cysteine and Histidine Rich
PPL	Paired Posterior Lateral
PPM	Paired Posterior Medial
PKA	Protein Kinase A
PKB	Protein Kinase B
PSD	Post-Synaptic Density
Rac1	Ras-Related C3 Botulinum Toxin Substrate 1
Rho	Ras-Homology
RhoGAP18B	Ras-Homology GTPase Activating Protein 18B
ROI	Region of Interest
Rsu1	Ras suppressor 1
RTK	Receptor Tyrosine Kinase
SAGE	Study of Alcoholism: Genetics and Environment
scb	Scabrous (integrin mutation in alpha receptor subunit)
SEM	Standard Error of the Mean
shi^{ts}	Shibire temperature sensitive
SNP	Single Nucleotide Polymorphism
S6K	S6 Kinase
S6k-P	S6 Kinase-Phosphorylation
slo	Slow Poke
ST-50	Time to 50% Sedation
STM	Short-Term Memory
Syn	Syntaxin
SYX1A	Syntaxin-1A
Tao	Thousand and one Protein
TOR	Target of Rapamycin
TSC	Tuberous Sclerosis Complex
TrpA1	Transient Receptor Potential A1
UAS	Upstream Activation Sequence
vol	*volado*
VEGF	Vascular Endothelial Growth Factor
VTA	Ventral Tegmental Area
whir	white rabbit
WT	Wild-type

CHAPTER 1: Overview of alcoholism

A. Introduction

Alcohol's disinhibiting and pleasurable effects have been enjoyed by humankind for millennia, and its still recreationally consumed today. However in some individuals, alcohol-use leads to the development of alcoholism, an affliction with severe consequences for individuals and society. For instance, the World Health Organization (WHO, 2004) ranks alcohol use as the third most serious risk factor for the loss of health, representing 4–6% of the global burden of disease and injury for all ages (Mathers et al., 2008; Rehm et al., 2009; Navarro et al., 2011). In the United States alone, about 18 million people have an alcohol use disorder (AUD) with more than 105,000 annual deaths ascribed to its use (Rehm et al., 2009). In European countries, the prevalence rate for alcohol binge drinking among adolescent teenagers reaches about 32%, and correlates with a particularly bad prognosis for future adult alcohol dependence (Hibell et al., 2003). Overall, AUDs are extremely costly to individuals and society in terms of productivity, morbidity and mortality.

Although treatment for many patients with alcoholism is effective, there are substantial proportions of patients who do not respond to the therapies available. A major problem jeopardizing treatment from alcoholism is the addictive nature of the illness itself. Alcoholism is a relapsing disorder characterized by (1) compulsion to seek and take alcohol, (2) loss of control in

1

limiting chronic intake, and (3) motivation to resume alcohol intoxication to avoid the emergence of a negative emotional state (i.e. dysphoria, anxiety, irritability) during a period of abstinence/withdrawal (Koob and Volkow, 2010). Early abstinent AUD patients report significant difficulties with alcohol craving (or wanting) and struggle with relapse urges (Seo and Sinha, 2014). Although our understanding of how alcohol-use transitions to difficulties with craving is limited, studies show that this transition is a result of the brains need to adapt to alcohol's chronic effects (Koob and Volkow, 2010). Alcohol initially has an inhibitory effect on the CNS. However, over time, the brain counteracts this inhibitory effect by enhancing excitatory (e.g. glutamate), and suppressing inhibitory neurotransmitter systems (e.g. GABA, (Manzo-Avalos and Saavedra-Molina, 2010)). The brain thus adapts to the chronic effects of alcohol with changes in different brain regions proposed to affect the successive development of alcohol dependence.

Initially, acute alcohol intoxication generates feeling of pleasure and reward by promoting dopamine transmission in the mesolimbic dopamine system. Elevated dopamine levels in the mesolimbic pathway respond to rewarding stimuli such as food, sexual arousal and other drugs of abuse. The reward pathway begins in the ventral tegmental area (VTA), which contains dopaminergic cell bodies and connects to the ventral striatum (VS, also known as the nucleus accumbens). The VS is composed of gamma aminobutyric acid (GABA, a inhibitory neurotransmitter) medium spiny neurons and also receive

input from the glutamatergic neurons of the hippocampus, amygdala, and prefrontal cortex (PFC). Preclinical studies show that acute alcohol administration stimulates DA release and GABA activity in the ventral striatum to trigger positive reinforcement/reward learning (Rewal et al., 2009; Seo and Sinha, 2014). With continued alcohol intake, neuroadaptive changes in the brain decreases dopaminergic neurotransmission in the reward circuit (VTA-VS) promoting tolerance and increased alcohol self-administration (Koob et al., 2004). The dorsal region of the striatum (dorsal striatum, DS) is also engaged in action of initiation and habit learning which promotes impulsive decisions of alcohol-seeking behavior leading to loss of control in limiting compulsive alcohol intake (Kahnt et al., 2009). Therefore, a sensitized striatal DA system increases alcohol salience and the vulnerability of alcohol-dependent patients, which positively reinforces craving (or wanting) and alcohol seeking behaviors.

With continued alcohol self-administration, the reward state is decreased. Other interconnected brain regions involved in stress and arousal become sensitized and may play a role in the emergence of a negative emotional state that motivates individuals to crave alcohol. Alcohol-dependent patients frequently report negative affects such as anxiety, and dysphoria during alcohol withdrawal. In early abstinent AUD patients, high uncontrollable alcohol craving is often accompanied by anxiety, tremors, high blood pressure, hallucinations (visual, auditory, or tactile), seizures, or delirium tremens, which are caused disruption of the hypothalamic-pituitary axis system and an increase in stress hormones such

as cortisol (Manzo-Avalos and Saavedra-Molina, 2010). These symptoms of negatively reinforced craving in early recovering AUD patients relates to their inability to regulate emotional distress, which is induced by increased activity of the amygdala. The amygdala, a part of the limbic system that reacts to negative emotions and fearful stimuli, establish associations between alcohol cues (visual, auditory, or tactile) in the environment and high alcohol craving in AUD patient (Koob et al., 2004; Namburi et al, 2015). The hippocampus, a part of the brain required for context/spatial dependent learning and memory, along with the amygdala could also pair alcohol's euphoric memories with certain context and cues in the environment that make patients struggle with withdrawal (Rao et al., 2015). Lastly, the PFC, which provides executive control over choices made in the environment, is severely hindered in by chronic stimulation and sensitization of the reward circuit. Dysfunction in the PFC is therefore detrimental to early recovering AUD patients as it impairs regulation of negative emotional states (i.e. the amygdala) inevitably leading to relapse despite negative impacts on their mental and physical health (Seo and Sinha, 2014). Taken together, neuroadaptive changes induced by chronic alcohol drinking in distinct brain regions result in poor treatment outcomes, perpetuates compulsive alcohol craving, and increases alcohol relapse risk in AUD patients (Koob et al., 2004; Koob and Volkow, 2010).

To date, the mechanism of action of alcohol is still unknown. One of the most alluring and accepted of theories is the effect of alcohol on *N*-methyl-D-

aspartate (NMDA) and GABA receptors at synaptic circuits. Studies suggest that acute ethanol exposure blocks the binding of glutamate and glycine to the NMDA receptor (Möykkynen and Korpi, 2012), thus inhibiting the release of neurotransmitters, such as serotonin, acetylcholine, dopamine, noradrenaline, endorphin, encephalin, endocannabinoids and neuropeptide Y (Crabbe et al., 2006; LeMarquand et al., 1994; Perra et al., 2008; Thiele et al., 2002). Additionally, many of the adaptive responses to chronic ethanol exposure in the brain such as tolerance, increased hyperexcitability, and alcohol dependence can be ascribed to compensatory changes in NMDAR activity, which also regulates synaptic plasticity (Carpenter-Hyland et al., 2004; Roberto et al., 2004; Ron, 2004; Woodward, 2000). Unfortunately, alcohol's action on these neurotransmitters and ion channels do not completely recapitulate the long-term effects seen with repeated alcohol exposures. Also, due to the myriad of neurotransmitters and neuromodulator systems affected by alcohol, the efficacies of current pharmacotherapies targeting alcohol dependence are limited (Seo and Sinha, 2014). Thus, a better understanding of the physiological and molecular pathways affecting alcoholism would give more insights into the long-term effects of alcohol and possible treatments for AUD patients.

B. Heritability of Alcoholism

Although the end-point of alcoholism is the same, progression to alcohol dependence is distinct for each individual with some returning to earlier stages of

the disease with varying frequency and intensity (Rao et al., 2015). The fact that people from similar environments often differ in their pattern, frequency and intensity of alcohol use underscore the role of genetics in the development of AUDs (Enoch and Goldman, 1999). Twin, adoption and sibling studies show that genetic vulnerabilities are directly responsible for some of the interindividual differences observed in risk for AUDs (Goldman et al., 1993). A Finnish twin study evaluated the use and abuse of alcohol in 879 monozygotic (MZ) and 1940 dizygotic (DZ) pairs of twins estimating the heritability of the various drinking habits, such as frequency and density of alcohol intake, to be at 0.36–0.40 (Kaprio et al., 1987). A Colorado sibling/twin/adoption study (n = 1000) of alcohol-initiation, -use, and -problem use found a weak to moderate heritability for alcohol initiation or frequency of alcohol use, but a substantial heritability (0.78) for problem use in adolescents. Data analyses from biological and adopted siblings in the same study showed a three- to five-fold increased genetic risk for developing alcoholism (Rhee et al., 2003). Overall, genetic studies have estimated the heritability of alcoholism to be around 40–60% (Gelernter et al., 2009; Dick et al., 2006). Therefore, studying genes affecting alcoholism will aid our understanding of the molecular mechanisms leading to alcohol abuse in humans.

C. Studying genes affecting Alcohol Use Disorders

Alcohol heritability studies demonstrate that genes do influence risks of developing alcoholism (Kaprio et al., 1987; Rhee et al., 2003). Like most

psychiatric disorders, alcoholism is a complex disorder where multiple genes are involved in different dimensions of its drinking behavior, and show no obvious pattern of Mendelian transmissions (Goldman et al., 2005). Identification of a single gene that accounts for a large amount variance contributing to the risk of alcoholism is thus very challenging. Although the precise nature of these risk factors is still unknown, genes involved in alcoholism express a common behavioral signature. Alcoholism can be defined as an illness of abnormally intense alcohol seeking behavior, which over time leads to uncontrolled compulsion to drink despite negative consequences, tolerance and relapse (American Psychiatric Association, 1994). Researchers have thus turned to endophenotypes as a way of dealing with the substantial heterogeneity involved with alcohol dependence. Endophenotypes are defined as the measurable intermediates between an observed disorder and the biological processes responsible for the manifestation of that disorder (Gottesman & Gould, 2003). Rationale for use of endophenotypes is if the clinical phenotypes associated with a disorder are very specialized and straightforward than the disease as a whole, then the number of genes required to produce variations in these traits are reduced and easily identified (Schumann, 2007). Endophenotypes would ideally have monogenic roots but it is possible that many would have polygenic bases themselves. For instance, several lines of evidence indicate that the level of response to sedating ethanol doses in humans is predictive of future risk of alcoholism (Schuckit et al., 2004). That is, a 20 year old individual displaying

acute resistance to ethanol intoxication is four times more likely to become an alcoholic 10 years later (Schuckit et al., 2004; Schuckit, 1994). Therefore, acute ethanol sedation can be a simple endophenotype examined in humans or different model organisms to aid identification of genes that contribute to alcoholism since responses to the sedating effects of alcohol are conserved from invertebrates to vertebrates.

Despite the promise of current human genetic methods, such as genome-wide association studies (GWAs), RNA- and whole exome- sequencing approaches (Sanders et al., 2012), they often fail to identify disease susceptibility genes with great certainty. This is due to the fact that validity of genes identified through these methods relies on their statistical power, which is contingent on the amount of subjects in a given study. Animal models thus remain highly useful for coordinated analyses of genes/molecular pathways, brain circuit functions and behavior. However, translational approaches that utilize model systems in conjunction with GWAs and other human clinical studies can help to accelerate the validation of responsible genes, and our understanding of their functional relevance in AUDs (Schumann, 2007). Genes identified in animal models and then tested in human association studies will be more easily validated thanks to fewer statistical comparisons necessary compared to unbiased human GWAs. Using this translational approach, I show in my dissertation that *Drosophila melanogaster* (Vinegar/fruit fly) can be utilized as a model system to identify genes involved in alcoholism that are also functionally relevant in humans. I also

show that genes regulating the actin cytoskeleton are required in dopamine-influenced neuro-circuits (See chapter 8 & 9) to control different aspects of alcohol-induced behaviors ranging from acute sedation to experience dependent alcohol preference/consumption in flies and mammals.

Before discussing my dissertation results, I will first introduce how alcoholism is modeled and studied in *Drosophila* (Chapter 2). I will outline/review genes identified to affect alcoholism in both flies and mammals with a later emphasis on the actin cytoskeleton (Chapter 3).

CHAPTER 2: Drosophila as a model organism

* This chapter has been accepted and published in the Biological Research on Addiction by Elsevier in 2013. I co-wrote the manuscript with Dr. Rothenfluh.

A. Criteria for modeling alcohol addiction

Over the years, researchers studying alcoholism have attempted to develop suitable animal models and came to the realization that these models are limited in their ability to show some aspects of human alcohol abuse. Although the behavioral response to alcohol is evolutionarily conserved, human psychosocial and cultural variables cannot be incorporated into animal models. Also, most animal models examined, do not like the taste of alcohol; despite its reinforcing properties, and researchers have gone to great lengths to disguise its taste with a palatable solution. Therefore, researchers proposed 3 criteria an animal model must ideally satisfy to study alcoholism, which are:

1. The animal must self administer alcohol to pharmacologically significant amounts irrespective of its caloric value, taste or smell.

2. Tolerance to ethanol should be observed in terms of performance to the same dose of ethanol and after periods of chronic exposure.

3. Physical and psychological dependence to ethanol should develop after a period of continuous consumption. Although the variable of human-physical dependence cannot be totally modeled, if ethanol maintains operant responding after a period of forced abstinence in models, some degree of behavioral dependence or relapse can be assumed to have occurred.

Using these criteria, mammalian models such as mice, rats and human studies have provided significant insight into mechanisms underlying alcohol use disorders. However, one invertebrate model organism better suited for looking at the genetic component of alcoholism is *Drosophila melanogaster* (fruit fly). In this chapter, I will cover the utilization of the fruit fly as a model to study alcohol responses

B. Advantages of Drosophila

Vinegar flies have been used for over a 100 years as a model organism to study the laws and mechanisms of heredity, and basic biology (Rubin and Lewis, 2000). The main reasons why Thomas Hunt Morgan's research group decided to introduce Drosophila as a genetic model organism in their laboratory over 100 years ago were:

1. Flies are easily and inexpensively maintained in the laboratory.

2. Their generation time is short, requiring less than 2 weeks to go from a freshly laid egg to a reproducing adult.

3. A single female's offspring is numerous, reaching over 500 eggs in a lifetime with a maximal rate of one egg per 30 min.

In addition, the salivary gland chromosomes of flies happen to be visible with the light microscopes available at the times (Rodan and Rothenfluh, 2010). This allowed Drosophila researchers to isolate and characterize a large number of defined copy number variants, known as deficiencies and duplications. These, as well as other chromosomal rearrangements isolated, helped immensely in

establishing genetic maps, and proved to be invaluable for fly stock maintenance (Rubin and Lewis, 2000).

C. Tools for forward Genetics

Forward genetics is the approach where a heritable phenotype is isolated, and then researchers work toward identifying the altered gene(s) causing that phenotype. This has been the traditional approach in flies, and to increase the chance of recovering flies bearing a phenotype, the mutation rate is commonly increased by artificial means. X-rays were discovered to cause mutations in flies, and were originally a popular mechanism to induce changes in DNA (often times complicated genomic rearrangements) (Sturtevant, 1967). Chemicals, modifying DNA bases, and causing mostly single base pair changes, have also been popular. Recently, biological agents, such as transposable elements (P-element) have been widely used. These allow fast identification of the genes affected, and although their DNA insertion preference is not random, different elements are available, with different insertion bias, thus allowing a wide sampling of potentially affected genes. Finally, the *Drosophila* genome has been fully sequenced, annotated, and shows extensive gene conservation with humans, though with less genetic redundancy (Adams et al., 2000). The fact that an estimated 70-80% of human disease genes have conserved orthologs in *Drosophila* (Chien et al., 2002), has confirmed the vinegar fly as an excellent genetic model organism, including for probing and deepening our mechanistic understanding of human diseases (Pandey and Nichols, 2011).

D. Tools for Reverse Genetics

Testing the hypothesis that a given gene of interest is involved in alcohol responses, i.e. going from gene to phenotype has also become increasingly feasible in flies (Rubin and Spradling, 1982). Currently, more than 90% of Drosophila genes harbor a mutation that can be ordered as a fly strain from one of the numerous stock centers around the world. In addition, most genes' function can be reduced with RNAi strains, specifically targeted to knock down a specific gene's mRNA levels. Again, fly strains carrying these RNAi constructs are publicly available from stock centers. The gold standard in fly genetics is to restore the function of a mutated gene by introducing a transgene carrying a wild-type version in the mutant background. This allows researchers to ask whether the observed mutant phenotype is rescued toward wild type. Transgenesis has been available in the fly for 30 years, and has become an invaluable tool not only for rescue experiments, as mentioned above, but also to study gene function in space and time (see next section). Lastly, in the past 10 years the techniques for homologous recombination have been introduced, and improved, allowing for the precise knock out of a given gene (fragment) in its endogenous genomic location. Thus, while going from phenotype to gene has been the traditional approach in Drosophila, it is now equally feasible to do hypothesis testing, and study predetermined genes, or whole gene families, for their involvement in alcohol responses.

E. Gene Manipulation in Space and Time

Transposable elements can be engineered to a researcher's specification, and 25 years ago a transposon carrying the bacterial gene lacZ was randomly inserted in many different genomic locations in Drosophila (Rubin and Spradling, 1982). Surprisingly, lacZ was expressed in many different patterns in space and time, reflecting the endogenous expression patterns and dynamics of the gene wherein the transposon had inserted. These so-called enhancer trap lines have become the basis for the development of gene-traps, both in flies and mammals. Large collections of fly strains have been made that carry the Gal4 gene in many random locations, or under the control of a specified promoter, and these lines express Gal4 in many different anatomical regions in the fly (Brand and Perrimon, 1993). The yeast transcriptional activator Gal4 binds directly to the upstream activating sequence (UAS), which is not present in flies. The first component, Gal4, in the Gal4/UAS binary system allows for testing of the spatial, and anatomical requirements in a process, while the transgene carrying UAS can be used, for example, to determine whether restoration of gene's expression rescues that gene-mutant's phenotype (Brand and Perrimon, 1993). In addition, the Gal4/UAS systems allows testing of questions such as which domains are required in a protein to carry out its normal function, or it can be used to specifically interfere with neuronal function, i.e. to make action potentials more or less likely, or even to abolish, or elicit them on demand. With its versatility, the Gal4/UAS system has revolutionized the way genes can be manipulated, and it is increasingly being used to precisely manipulate CNS function in the behaving

animal too. Temporal specificity can be achieved by using a temperature-sensitive Gal4 repressor called Gal80[ts] and shifting the flies from the permissive to the restrictive temperature during a particular time period (Kaun et al. 2012). Other strategies for controlling target gene expression at specific times in *Drosophila* have been developed, including hormone-mediated GAL4 activation approaches that include a chimeric GAL4, GAL4-ER, and GeneSwitch systems Osterwalder et al., 2001, Nicholson et al., 2008; Roman et al., 2001). In these systems the addition of exogenous molecules such as diethylstilbestrol (DES) or β-estradiol increases GAL4-ER activity (Han et al., 2000) and mifepristone (RU486) induces the GAL4 activity of GeneSwitch (Roman et al., 2001). Both systems transactivate target gene expression via the *UAS* when transgenic flies consume fly food containing hormones. Although the GeneSwitch system is more common now and GeneSwitch-enhancer trap lines have been developed in recent years, it lacks the rich spatial variety that traditional GAL4 drivers provide.

F. Alcohol and Drosophila

Initial studies of Drosophila and alcohol have focused on alcohol-induced toxicity, how flies can become resistant to it, and how they can utilize ethanol as a food source. Much of this was driven by two factors: First, the belief that ethanol attracts flies and is a relevant food source, given the presence of ethanol in rotting fruit. Flies can indeed efficiently utilize ethanol as a food source, but their attraction to it is tenuous (see below), while they are unquestionably attracted to yeast. Second, the major ethanol catabolizing enzyme alcohol

dehydrogenase (Adh) was one of the first proteins where two distinct variants could be followed with early biochemical methods (David et al., 1976). In the last 15 years, Drosophila has become an accepted model organism to study alcohol-induced behaviors, and it satisfies the three criteria for modeling alcohol use disorders outlined above. The Drosophila specific behaviors fitting these criteria, and the ways they are induced and measured are described below. The last 15 years have shown that genes and biochemical pathways that contribute to human alcoholism, such as the dopamine system, are conserved in flies (See Chapter 8). For example, cheapdate was the first mutation affecting behavioral alcohol responses flies that was extensively studied (Moore et al., 1998). The mutated gene in cheapdate is the fly ortholog of the human pituitary adenylate cyclase-activating polypeptide (PACAP) (Maas et al., 2005; Moore et al., 1998). *cheapdate* is involved in cyclic AMP/ protein kinase A signaling, and in mice, mutations altering cyclic AMP signaling, including a mutation in PACAP, have subsequently been shown to alter the response to alcohol in the affected mice. As a second example, the hangover gene is required for proper alcohol tolerance in flies, and the human orthologs ZNF699 was later found associated with human alcohol dependence in an Irish sib pair study (Riley et al., 2006; Scholz et al., 2005). Many more examples of mechanistic conservation have been found (See Chapter 3), thereby highlighting the usefulness of Drosophila in deciphering the genetic basis and mechanistic underpinnings of behavioral alcohol responses.

Over the years, a number of ways have been used to expose flies to

varying doses of ethanol. The most widely used ways are described below, together with the assays developed to quantify the behavioral responses:

a. Ethanol as an Odorant

One major way how flies perceive the world is through olfaction. The olfactory system of Drosophila is one of the best-understood sensory perception and processing circuits (Waddell and Quinn, 2001). On a molecular level, a number of active ligands are known for many odorant receptors. To date, this is not the case for ethanol, and while many publications describe behavioral responses to ethanol odor, no olfactory receptor has been found that shows significant responses to ethanol. Still, sensory response assays testing the attractive, or repulsive quality of ethanol odors have been used repeatedly, and they fall into two categories. One method to measure the attraction to ethanol is with the olfactory trap assay. Here, flies are lured by the smell of ethanol into a vial sealed with a funnel, acting as a one-way valve (Reed, 1938). Through this method, the percentage of flies attracted by a given odor can be measured, or if two traps are presented at the same time, a preference index (PI) can be calculated. An olfactory trap assay is commonly run over many hours, because the trap presents a small volume within a much larger receptacle. A second, more acute response to alcohol can be measured in a Y- or T-maze. As the name implies, these are Y- or T-shaped mazes where flies are motivated to run toward the maze's diverging path and then decide between one of two directions. Here, they are given a choice to enter an ethanol-containing tube or take the

other direction (empty or different odorant). This assay allows the assessment of flies' preference for the smell of ethanol within minutes.

Oviposition preference is another assay that measures female flies response to ethanol in the food, or environment. This is done by measuring their propensity to lay their eggs onto ethanol-containing food compared to control food, either in direct competition as a preference value, or in absolute numbers of eggs laid. While such assays have been used to show an effect of ethanol on egg laying, it is not clear what sensory modality is actually engaged to detect the alcohol (McKenzie and Parsons, 1972).

b. Exposing Flies to Vaporized Ethanol

This is the most common method used to expose flies to ethanol. In this assay, ethanol is vaporized by bubbling air into a gas-washing bottle containing ethanol. The air stream of vaporized ethanol is then combined with a second air stream, humidified with pure water. The combined, ethanol–air stream then flows at predetermined, but adjustable rates into an exposure chamber containing flies (Wolf et al., 2002). This way, flies will continuously be exposed to alcohol through their tracheal system for the duration of the exposure. Similarly, a defined amount of ethanol can be put onto a cotton ball contained within a fly exposure vial. Through passive vaporization, flies get exposed to ethanol, and their responses can be measured. The response of flies to vaporized ethanol has three phases: First, they show vastly increased locomotion due to an olfactory startle reflex. This response is quick acting, within seconds, and brief in duration, less than a

minute. Even though ethanol flow continues, the startle response habituates and flies become more stationary again. During the startle response, no significant increase in hemolymph ethanol levels can be measured, and surgical removal of the main olfactory organ, the antenna, abrogates this response. Thus the initial startle response is a response to peripheral sensation. Second, by 5–15 min into the exposure, depending on the ethanol flow rate, flies become hyperactive again. At this time, the ethanol concentration in the hemolymph has reached 15–30 mM (corresponding to a blood alcohol concentration of 0.7–1.4 g/l). Since this alcohol-induced hyperactivity is also seen in flies without antennae, it is thought to be a reaction to increased ethanol levels in the brain. Third, as exposure continues, flies stop moving, become progressively sluggish, and then fall over, unable to right themselves. When the ethanol exposure is terminated, the unresponsive, sedated flies will recover within 10–20 min (Wolf et al., 2002). See the next section for a detailed description of the behavioral assays measuring those responses.

c.   Injecting Ethanol into Flies

Given the small size of a fly, about 2 ml in volume, it would seem rather daunting to directly inject ethanol into flies. However, direct injection offers the advantage of knowing the exact exposure dose, as well as the precise time of the discrete exposure. Using this method, scientist reported the involvement of GABA receptors in acute response to alcohol (Dzitoyeva et al., 2003). Other than a requirement for micromanipulation equipment, two additional disadvantages of

direct injection are the low throughput and a lag time before behavior can be assessed, since flies obviously have to be restrained at the time of injection. Nevertheless, flies have been injected directly with ethanol into their abdomen, and time to sedation was measured. A similar, but more unusual, method involves delivery to individual headless flies. In this method, a droplet of ethanol is applied to the severed neck of flies' bodies onto the thoracic ganglion (Manev et al., 2004). Such headless flies continue to exhibit locomotion movements, and even grooming behavior. Although its obvious limitations, this paradigm has been successfully used in studies of alcohol and other centrally acting drugs.

d. Alcohol Ingestion in the Food

Mixing alcohol in food is the traditional way that researchers have studied ethanol response and toxicity. To test whether a fly strain is resistant to alcohol's toxic effects, researchers add alcohol to the culture medium serving as the flies' food (Geer et al., 1993). Such analyses allowed for the isolation and study of fly resistant strains in laboratory (David and Van Herrewege, 1993). For instance, resistant strains were obtained by selectively breeding flies that survived exposure to high alcohol levels in their food. To measure flies preference for ethanol, researchers usually quantified the mouth or proboscis print indented on food by flies or coloring the food and measuring how much the flies ate. This method has the disadvantage of not precisely measuring the amount of alcohol consumed. Also death by starvation can be easily misconstrued as ethanol

toxicity. Fortunately, the introduction of the CAFÉ assay has made this a problem of the past (Ja et al., 2007, described in the preference and reward section).

G. Ethanol-Induced Behaviors and Responses

As mentioned above, the behavioral response of alcohol in mammals are evolutionarily conserved in flies. Flies when exposed to low doses of ethanol exhibit disinhibitory behavior observed as an increased spontaneous locomotion called hyperactivation. On the other hand, flies are sedated at high doses (Corl et al., 2006). Additionally, Researchers have also shown that the internal ethanol concentration is about 20 mM at times of hyperactivity (corresponding to a Blood Alcohol Concentration (BAC) of 0.09%) and 45 mM at the time of sedation (corresponding to 0.21%, Rodan et al., 2010). This suggests that they also show the same behavior similar to humans at these concentrations. Furthermore, there evidence in humans as well as mammalian models that sensitivity to acute ethanol-induced motor impairment correlates inversely with ethanol consumption and risk of abuse, and that the same genes can influence both types of behavior (Schuckit 1994; Kurtz et al., 1996; Thiele et al., 1998; Hodge et al., 1999). Therefore, studying acute ethanol exposure in flies would help isolate genes affecting mediating this behavior in humans. The following assays are used to study these alcohol behavioral responses. In this section, I will review the numerous behavioral responses to acute, or repeat exposure to alcohol, and the assays that have been developed to quantify them.

a. Acute Behavior Responses

Sensory Response to Alcohol

Vinegar flies are easily observed in nature around rotting and fermenting fruit (Dudley, 2002; Hoffmann and Parsons, 1984; Rodan and Rothenfluh, 2010). Given that in the nineteenth century D. melanogaster once was called Oinopota cellaris, literally meaning the wine-drinker down in the cellar, it seems only natural to assume that they love ethanol. However, the literature on olfactory preference for ethanol is somewhat contradictory. Even when only focusing on papers that indicate an attraction to ethanol vapors, the potency of ethanol as an attractant is nowhere near the potency of acetic acid, for example, another component of fermenting fruit. This is also true for the ability of ethanol to stimulate/attract female flies' oviposition. Again, acetic acid proves to be a much more potent odorant. Thus it seems that Drosophila's common name "vinegar fly" is indeed appropriate, and need not be changed.

Alcohol-Induced Disinhibition

As mentioned above, the behavioral responses to alcohol in mammals are evolutionarily conserved in flies. Given low doses of ethanol, flies display markedly increased locomotion. Early assays used to study ethanol-induced hyperactivation involved manual counting of flies' line crossing, and using the inebriactometer to measure how often flies break an infrared beam when exposed to ethanol. While these assays give some information on ethanol-

induced spontaneous locomotion, the temporal and spatial resolution of these analyses are not very detailed. Therefore, a video-based locomotion tracking system, using the booze-o-mat was developed (Wolf et al., 2002). The "booze-o-mat" allows for filming of eight parallel tubes of flies being exposed to ethanol vapor. Video tracking software then determines the average locomotion speed of individual, or groups of flies as a function of exposure duration (Wolf et al., 2002). The analysis has sub-second resolution, and can even extract parameters such as heading, or turning angle of individual flies. Only the introduction of this tracking method has allowed for a clear subdivision of the behavioral response to alcohol into the three phases mentioned above. However, it is noteworthy that the loss of postural control and loss of locomotor activity (akinesia) cannot be distinguished with most video tracking setups, since akinesia precedes loss of postural control by a few minutes. Alcohol's effect on locomotion is by increasing walking speed and the duration of walking bouts, while bout frequency (i.e. the likelihood that flies will initiate walking) remains unchanged. As in mammals, this motor-stimulant activity of ethanol requires the biogenic amine dopamine (Wolf et al., 2002).

Alcohol-Induced sedation

When flies are exposed to a substantial dose of ethanol, they stop moving, start to lose postural control, and then fall on their backs unable to get back up. In a classic, but less popular setup these days, flies are exposed in the

inebriometer, a 125-cm long vertical column containing a series of angled mesh baffles. Flies are placed at the top of the column, where they tend to stay if exposed to humidified air only. As ethanol vapor flows through the cylinder (Wolf et al., 2002), they lose their postural control, fall down the baffles, and eventually elute from the column. A population of flies elutes from the column as a function of exposure time, and the mean of that exposure time is a function of the ethanol to airflow rate ratio, i.e. ethanol dose. This Mean Elution Time (MET) was the first behavioral ethanol response found to have a strong genetic component, and it remains a widely used measure, even though it is unclear how it relates to active ethanol ingestion preference (see below). Since the inebriometer is a big and unwieldy apparatus, not many laboratories utilize it routinely. Some laboratories use the "booze-o-mat" to visually inspect for loss of righting. Others simply expose flies to ethanol vapors wafting off a cotton ball, in a regular fly food tube, and then measure the time until the flies lose their ability to either right themselves (loss of righting) (Maples et al., 2011). Such a simple in-tube assay also reveals the same differences observed in the inebriometer. In some studies, ethanol induced-loss of locomotion activity has been used as a measure for sedation. It is worth noting that at an intermediate dose of alcohol, the loss of locomotion activity precedes the loss of postural control by several minutes, and some fly strains have been observed to dissociate loss of locomotion from loss of postural control. Therefore, alcohol-induced locomotion should not be regarded as sedation and loss of postural control. To ascertain ethanol sedation, exposed

flies can be inspected visually to determine whether they are still standing or lying on their backs (Rothenfluh et al., 2006; Corl et al., 2009)**.** Challenging flies with a mechanical stimulus can also be used to determine how many flies fail to regain their posture, thereby measuring sedation. When sedated flies are placed into humidified air, they will recover within 10–20 min. This can be measured as median recovery time, analogous to ethanol-induced sleep-time in rodents. Both ethanol-induced sedation, and recovery from it can be assessed by visual inspection, or by lightly tapping the vial containing the flies to test for loss of righting. A more demanding behavioral test that is sometimes used to test for inebriation is startle-induce negative geotaxis (Bhandari et al., 2009). When flies are mechanically stimulated, they respond by rapidly climbing upward, and this response is affected when flies are inebriated.

b.   Responses to Repeat, or Chronic Ethanol Exposure

Alcohol-Induced Disinhibition

As mentioned above, flies show increased locomotion with low levels of ethanol exposure. This response can be altered by a prior alcohol exposure. If flies are grown in 5% ethanol-containing food for example, they later show increased ethanol-induced hyperactivation as adults. Similarly, a single sedating dose of ethanol also increases the amplitude and duration of subsequent ethanol-induced hyperactivation 4 h later. A striking example of ethanol-induced disinhibition is displayed after repeat daily exposure. Starting on the second day, and increasing through the fourth day of exposure, 10–50% of exposed males

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start to court other males. Normally, young males learn to inhibit their courtship toward other males, but in this case, repeat ethanol exposure causes an inappropriate disinhibition toward other males, as well as females. Though mating performance, as measured by successful copulations, are actually decreased. Numerous fly species initiate courtship immediately, even toward inanimate objects of the right size. *Drosophila melanogaster* (and other species) have evolved an inhibitory circuit, including a group of neurons called the median bundle. Therefore, repeat ethanol exposure may lead to courtship disinhibition by silencing these inhibitory median bundle neurons.

Tolerance to the Sedating Effects of Ethanol

Tolerance is generally measured in flies as the acquired resistance to ethanol-induced sedation by a prior ethanol exposure. It is one of the criteria reported by several studies to contribute to the development to substance abuse. In Drosophila, researchers focus on functional tolerance, or tolerance acquired without any changes in alcohol absorption or metabolism. Expression levels of Alcohol dehydrogenase are very responsive to alcohol exposure in larvae, but in adults, this is not observed. Therefore adult behavioral tolerance is thought to be a CNS-mediated adaptation, and indeed, ethanol absorption and metabolism during a second exposure are unchanged after a sedating alcohol exposure (Scholz el al., 2000).

There are two types of ethanol tolerance, rapid and chronic, that have

been studied in Drosophila. Rapid tolerance is induced with multiple, distinct repeat exposures that cause sedation. The change in mean sedation or elution time after a first exposure is used to measure tolerance. This is often given as an absolute difference in minutes, which is however ethanol dose dependent. A better way is to indicate the relative tolerance, compared to the first exposure, which is less dose dependent. Rapid tolerance is often measured after two exposures, spaced 4 h apart. A more detailed kinetic analysis reveals that tolerance is maximal at 2 h after the first exposure with a 60% increase in mean elution time and declines rapidly to ~40% by 5 h. 5–25 h after the first exposure the level of tolerance declines only slightly, indicating that it is a fairly long-lasting phenomenon (the average lifespan of a fly is about 40 days). Because behavioral sensitivity or resistance to acute alcohol exposure and changes in tolerance are not mutually exclusive, the duration of ethanol exposure in fly strains with altered naive sedation-sensitivity when studying rapid tolerance has been called into question. For instance, is it appropriate to expose sensitive and normal flies to ethanol for the same duration, or should they be exposed until their times of sedation? This question could affect possible tolerance properties a fly strain might exhibit. Since rapid tolerance is dependent on a number of parameters, it is apparent that a tolerance dose–response curve should be obtained to truly ascertain that any tolerance defect observed is consistent irrespective of initial exposure dose (Scholz el al., 2000).

Chronic tolerance is induced with long-term subsedation doses of ethanol

exposure. Here, flies are exposed to a low dose of ethanol for a period of days (2 days normally) and then challenged with a high concentration of ethanol. As for rapid tolerance, the mean elution time of these flies are higher compared to those exposed to only air. The amount of tolerance induced by chronic exposure is slightly lower compared to a rapid or acute exposure, and its kinetics shows a steady decline over time. However, chronic tolerance is longer lasting, and can still be observed 48 h after the end of the ethanol pre-exposure (Scholz el al., 2000). Another noteworthy difference between chronic and rapid tolerance is the requirement for protein synthesis to acquire chronic, but not rapid, tolerance. So clearly two different mechanisms are participating in the establishment of acquired ethanol tolerance, also highlighted by the fact that they can be genetically separated.

c. Preference and Reward

Alcohol Self-Administration/Preference

Clearly, voluntary drinking is a hallmark of ethanol abuse. The establishment of the CAFE´ assay to study voluntary ethanol consumption is therefore a great step forward in the study of behavioral alcohol responses in flies (Devineni et al., 2009). As mentioned above, the CAFE´ assay allows for the precise measurement of ethanol-containing food consumed by flies over days. When given a choice between sucrose/yeast liquid food with, or without 15% ethanol, flies initially show a preference index (PI, where +1 is full preference, and -1 is full avoidance) of ~0.15, which increases over 3–4 days to 0.4. Two

additional experiments highlight that this preference shows addiction-like features. First, after 1 or 3 days of forced abstinence, the flies immediately went back to a PI of > 0.3 when given an ethanol choice again. This indicated that they remember preferring alcohol, and that they do not have to reacquire preference from an initial PI of 0.15. Second, when the ethanol-containing food was spiked with quinine, the flies would still acquire preference for this food, despite their initial avoidance of this bitter-tasting mixture (Devineni et al., 2009). Thus flies show relapse-like behavior, and they are willing to overcome adverse taste to ingest alcohol. It will be interesting to see how ethanol preference correlates with other alcohol-induced behavioral responses, and whether genes affecting one will also affect another response.

Since flies have been a long-standing model organism for genetic screens, (Bellen *et al.*, 2010; Rodan & Rothenfluh, 2010), researchers sought to improve on the high throughput limitation of the Café assay and developed a novel assay that measures the consumption preference index of individual flies in a fluorescence plate reader (See chapter 6).

Conditioned preference for ethanol reward

To test directly whether intoxicating doses of ethanol are rewarding to flies, a conditioned ethanol preference assay was recently developed (Kaun et al., 2011). In this assay, flies are initially exposed to two neutral odor cues, one of which is paired with a moderately intoxicating exposure to ethanol vapor. Flies

are later offered a choice between the two odors, and preference for the ethanol-associated odor is measured. Similar to mammalian conditioned place preference (CPP) models, this assay uses odor conditioned preference to assess the rewarding properties of ethanol intoxication. When flies have been trained to associate an odor cue with ethanol intoxication, they show initial aversion to the cue, which, within 12–15 h, transforms into a long-lasting preference (Kaun et al., 2011). The development of conditioned preference is dependent on the ethanol concentration; preference is induced only by exposure to moderate ethanol doses that induce locomotor hyperactivity. Conditioned preference is not induced by lower ethanol concentrations that fail to elicit behavioral changes or higher concentrations that cause sedation. Thus, flies seem to require an intoxicating, but not sedating, dose of ethanol for it to be remembered as rewarding. Remarkably, flies will endure electric shock in order to attain the cue associated with ethanol, indicating that they are willing to tolerate punishment to seek the drug (or, in this case, a cue that predicts the presence of the drug) (Kaun et al., 2011). This response is reminiscent of compulsive behavior such as impaired response inhibition observed in mammalian studies of drug reward. Furthermore, flies will endure a stronger shock intensity to attain a cue associated with ethanol than a cue associated with sugar, suggesting that the preference for ethanol is distinct from a preference for food reward (Kaun et al., 2011).

d.   Ethanol-Induced excitotoxicity

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Chronic alcohol consumption leads to neuropathology and neuronal death. In humans, the brains of alcoholics are reduced in weight and volume, and ≈10% of alcoholics develop a severe cognitive disorder, such as alcoholic dementia or Wernicke-Korsakoff Syndrome. Additionally, alcoholics' display diminished olfactory sensitivity, with one study finding that more than half of alcohol-dependent patients are hyposmic (loss of smell) (Rupp et al., 2003). Although the response of neurons to an ethanol insult is strongly influenced by genetic background, the underlying mechanisms are poorly understood. Drosophila melanogaster has been developed as a model to study genes contributing to excitotoxicity after ethanol exposure. French et al. (2009) developed a model showing that a single intoxicating exposure to ethanol causes non-cell-autonomous apoptotic death specifically in Drosophila olfactory neurons. This neuronal death is accompanied by a loss of a behavioral response to the smell of ethanol and a blackening of the third antennal segment. Using this model, French et al. (2009) isolated *Shaggy,* the human orthologous of Glycogen Synthase Kinase 3 β (GSK 3β), as a gene playing a molecular role in program cell death**.** This model can therefore allow for the investigation and screening of the genetic and molecular basis of ethanol-induced apoptosis (French et al., 2009).

H.   Large-Scale Approaches to Alcohol Studies in Flies

Since Drosophila is a small, inexpensive organism to cultivate, large-scale approaches seem especially well suited to investigate the responses to alcohol.

Forward Genetics

As mentioned above, forward genetics puts phenotype first, and the first indication that Drosophila may be a useful organism to study the genetics of ethanol responses came from selective breeding experiments. The inebriometer was used to selectively breed wild populations of Drosophila from the west coast of the United States. After 12 generations of selection, the mean elution time doubled, indicating that there is indeed a strong genetic component determining sensitivity to ethanol-induced sedation.

Traditionally, fly geneticists look for single-gene Mendelian inheritance, where one genetic change causes all of the phenotypic variance. In the laboratory, genetic changes are induced with chemical, or biological mutagens and then, many different flies, or fly strains are screened for their ethanol phenotype, and potential candidates are confirmed and followed up. Many research reports have described single-gene mutations that alter the behavioral responses of flies to ethanol. The most insightful of those, based on genetic screens, utilized transposable elements to disrupt random genes, and then screened a variety of alcohol responses for behavioral phenotypes. While chemical mutagenesis has also been performed, and has yielded strains with ethanol phenotypes, these studies have been less successful, simply because it has proven difficult to isolate the altered genes responsible for the observed behavioral phenotypes. In large part, this is due to the variability of ethanol-induced behavior, which can easily be influenced by different genetic backgrounds. And varying genetic backgrounds are

often specifically required to genetically map the chemically induced mutations. Thus without mapping strains the mutations cannot be located, but with mapping strains the phenotypes are too variable to accurately map. Hopefully in the future less variable mapping strains can be generated, or whole-genome sequencing approaches toward locating such mutations can be applied more gainfully.

Recently quantitative trait loci (QTL) type approaches have also become more popular. In a QTL-type analysis multiple loci are considered to additively contribute modest amounts of phenotypic variance to an observed phenotype. One such approach is for example to study 190 inbred strains isolated from the wild for their quantitative ethanol phenotypes. Since the full genomic sequence is known for all these 190 strains, it becomes feasible to do a genome wide association of phenotypic and genotypic variance. While the statistical power in such a relatively small sample is not that great, it is quite straightforward to subsequently test individual candidate genes, and whether mutations in only one of these candidate genes at a time also causes an alcohol phenotype. This has indeed been done for a subset of these 190 lines, and novel genes were isolated that affect ethanol responses.

Expression Profiling

To identify the mechanisms underlying alcohol dependence, several researchers have designed microarray experiments in Drosophila. Microarray, and more recently deep-sequencing, are methods to observe the expression levels of virtually all genes in an organism simultaneously. This genomic approach has been

applied in two ways for the study of fly alcohol responses. In one type of experiment, expression levels are examined in different genotypes, like the above mentioned 190 wild inbred strains that have different responses to alcohol. This approach allows for the correlation of an ethanol response phenotype, with the expression levels of genes, or co-regulated suites of gene. In a second approach, ethanol exposure itself is the variable, and genes are found that show changes in expression level upon acute, or repeat ethanol exposure. Both of these approaches have been successfully used to highlight individual genes involved in ethanol responses, or to suggest what signaling and functional pathways participate in fly alcohol responses.

I. Beyond the gene

Alcohol-induced changes in gene transcription may be highly relevant for the development of chronic tolerance, as it requires protein synthesis. Heritable changes in the transcription factors regulating target genes, or their promoters and enhancers could alter these ethanol-induced changes, thereby predisposing a given fly strain to an alcohol tolerance phenotype. Other mechanisms that can lead to altered gene expression are epigenetic changes. Modifications of the DNA-organizing chromatin structure such as histone acetylation, methylation, and ubiquitination can determine whether gene transcription can occur. These changes caused by mechanisms other than mutations or changes in DNA sequence can be heritable and are termed epigenetics. In mammalian models, histone modification has been shown to alter behavioral responses to drugs of abuse such as alcohol,

cocaine and others. Therefore, studies of epigenetics in Drosophila have also been developed. For instance, the slo gene, required for rapid tolerance, displays changes in transcription and histone acetylation upon exposure to alcohol. Epigenetic mechanisms are therefore contributing to the ethanol-induced behaviors both in flies and in mammals. Given the rapidly advancing technology, it should soon be possible to do large-scale surveys of not only transcript levels, but also protein levels, or even posttranslational protein modifications. That way, researchers will be able to identify additional regulatory events mediating the ethanol behaviors studied.

CHAPTER 3: Molecular mechanisms underlying ethanol-Induced behaviors in Drosophila

Drugs of abuse highjack circuits normally engaged by natural rewards such as food and sex. When used repeatedly, drugs elicit molecular and structural changes at the synapse that promote continued drug craving, and this can supplant almost all other of the animal's behavioral goals (Hyman, 2005). These experience-, and drug-dependent reorganizations of neural circuitry require adaptation of physiological/molecular signaling mechanisms that include various ion channels, enzymes, neurotransmitters, growth receptors and cytoskeletal element affecting the plasticity of the synapse. In chapter 2, I showed that a myriad of assays have been established to study various aspects of ethanol-induced behavior in Drosophila. These assays are simple, robust, and high-throughput, allowing researchers to conduct forward genetic screens to identify the underlying mechanisms leading to alcoholism. The genes identified in these screens have led to the characterization of diverse molecular and cellular processes that mediate ethanol-induced behavior in flies. Here, I describe genes shown to play a role in alcoholism in both flies and mammals.

A. Ion Channels and Neuromodulators

Drosophila has the same voltage gated- and ligand-ion channel receptors as mammals. 2 K^+ channels are associated with functional tolerance in

Drosophila. In mammals the KCNQ family of ion channels includes five members. Mammalian KNCQ2/3 ion channel produce M-current, which is inhibited by ethanol in rat dopaminergic VTA neurons (Koyama, Broadie, and Appel, 2007). In flies, the entire KCNQ family is represented by a single gene, dKCNQ. Like mammals, the dKCNQ channel is inhibited by ethanol. Reducing dKCNQ expression increased neuronal excitability, while increasing dKCNQ expression reduces neuronal excitability (Cavaliere, Gillespie, and Hodge, 2012). Furthermore, expression dKCNQ in dopaminergic neurons produces ethanol resistance.

A major regulator of neuronal excitability that have been implicated in ethanol-induced behaviors in flies are the gamma aminobutyric acid B ($GABA_B$) receptors. $GABA_B$ receptor activity promotes sensitivity to ethanol sedation but reduces rapid ethanol tolerance (Dzitoyeva et al. 2003). Like mammals, *Drosophila* $GABA_B$ receptors are metabotropically coupled to potassium channels, thereby inhibiting neuronal excitability due to potassium efflux (Mezler et al. 2001). Ethanol-induced change in the activity of big potassium (BK) ion channel is also implicated in rapid ethanol tolerance in flies (encoded by the gene *slowpoke* (*slo*), Cowmeadow et al. 2005, 2006). The fact that $GABA_B$ receptors and BK channels likely affect neuronal excitability in the same direction, but regulate tolerance in opposite ways, suggests that they may function in different subsets of neurons that exert opposing effects on behavior. Alternatively, BK channels may in fact enhance neuronal excitability by reducing the refractory

period or enhancing firing rates, allowing neurons to compensate for the depressant effect of ethanol during sedation (Atkinson, 2009). Ghezzi et al. (2011) showed that adult flies in alcohol withdrawal have increased CNS excitability and a significant increase in susceptibility for seizures. These withdrawal phenotypes are also dependent on *slo* gene expression underlying a hypothesis that tolerance and withdrawal symptoms arise from the same neuroadaptions leading to alcohol preference.

Early abstinent AUD patients show an increase in stress due to the sensitization of the HPA axis and the release of stress hormones such as cortisol. In flies, the neuropeptide corazonin is the invertebrate ortholog of gonadotropin-releasing hormone and is involved in various stress responses. Loss of corazonin activity correlates with stress resistance. Adult reduction of corzonin levels causes resistance to ethanol-induced sedation, while activation of these neurons leads to sensitivity. Interestingly, ethanol exposure is followed by an increase of corazonin levels within 15 minutes of exposure, highlighting the link between ethanol and stress responses (McClure and Heberlien, 2013). Lastly, stress/arousal arising from deprivation from natural rewards such as food and sex can increase the need to seek/want alcohol in flies. For instance, neuropeptide F (NPF), the human orthologs for NPY have been shown to regulate ethanol sedation in flies and mammals (Wen et al., 2005). Increased alcohol consumption in male flies after sexual deprivation is also on NPF expression in flies (Shohat-ophir et al., 2012), highlighting again the link between

ethanol and stress responses.

Like mammals, neurotransmitters such as dopamine (see chapter 4), octopamine (a biogenic amine thought to be the invertebrate analog of norepinephrine), and serotonin are also essential in regulating alcohol-induced behaviors in flies. Silencing of serotonin neurons in adult flies cause resistance to ethanol-induced sedation. Protein kinase C (PKC53E) also functions in serotonergic cells to mediate normal ethanol sensitivity with a knockdown of PKC53E in serotonergic cells leadind to ethanol resistance. Norepinepherine (octopamine) in flies is also required for the development of rapid but not chronic ethanol tolerance (Scholz, 2000; Berger et al., 2004). Similarly in mice, depletion of norepinephrine also suppresses the development of functional ethanol tolerance (Tabakoff et al., 1977) suggesting that the role neurotransmitter in flies and mammals are evolutionary conserved

B. Growth Factors and Receptors

The epidermal growth-factor receptor (EGFR) signaling pathway modulates ethanol's intoxicating effects in *Drosophila*. A report by Corl et al. (2009) demonstrated that *happyhour (hppy)*, Ste20 family kinase member negatively regulates the EGFR/extracellular signal regulated kinase (ERK) pathway to promote resistance to ethanol's sedating effects. EGFR signaling activates ERK. In flies, increasing EGFR or the *Drosophila* ERK homologue *rolled* in the nervous system produces resistance to ethanol sedation, while

neuronal knockdown of EGFR produce the converse, but not in the *hppy* mutant background (Corl et al., 2009). They continued to show that feeding EGFR inhibitors Erlotinib and Gefitinib, dramatically increased ethanol sedation in flies and decreased alcohol consumption in rats, further demonstrating the conserved role of EGFR in ethanol-related behaviors.

Insulin receptor (*InR*) signaling occurs by complex intracellular mechanisms that begin when secreted insulin peptides released binds its receptor at the cell's surface of a postsynaptic cell. Corl and colleagues (2005) demonstrated that *InR* signaling could also regulate ethanol-induced responses in the *Drosophila* CNS. For neuronal reduction in *InR* signaling causes sensitivity to ethanol sedation, while its activation results in resistance (Corl et al., 2005). However, a different study (Corl, Rodan, and Heberlein, 2005) using several more direct manipulations of the PI3K/Akt pathway demonstrated that this pathway promotes sedation sensitivity (Eddison et al., 2011). Nevertheless, a downstream target of the insulin receptor, P-S6K (phosphorylated form of S6K and substrate of the target of Rapamycin (TOR)), has been (pS6K) implicated as a marker of neuronal activation and affects ethanol sensitivity in flies (Acevedo and Rothenfluh, in revision).

C. Enzymes

Ethanol binds to the mammalian enzyme alcohol dehydrogenase (ADH) at a K_m constant of approximately 1mM (Negoro & Wakabayashi, 2004). Though moderately conserved, ADH shows species-dependent variation for binding to

different sizes of ethanol molecules, which is determined by the amino acid length adjacent to the binding site (Weinhold & Benner, 1995; Svensson et al., 2003). This variation in ethanol response sensitivity has been shown in human studies, where it was revealed that racially diverse populations with distinct naturally occurring polymorphisms in the ADH gene show different sensitivities to ethanol (Agarwal et al., 2000). Although, studies in larvae (David et al., 1976) and flies (Singh and Heberlein, 2000; Morozova et al. 2007) have revealed that are important for the toxic effects of ethanol, recent studies show that ADH also influences alcohol consumption/preference in the adult fly similar to what is observed in humans (Ogueta et al., 2010).

D. Transcription and Translation in Ethanol-Induced Behaviors

Gene transcription has also been implicated in ethanol response regulation in *Drosophila.* Transcriptional regulator encoding genes *Lim-domain only* (*dLmo*) and the *hangover (hang*) regulate ethanol-induced sedation and rapid tolerance respectively (Lasek et al., 2011a; Scholz et al., 2005). Although little is known of their downstream targets, *anaplastic lymphoma kinase* (*dAlk*) is the only identified downstream of *dLmo* (Lasek et al., 2011b). On the other hand, *hang* mediates oxidative stress sensitivity and heat shock induced ethanol tolerance via a mechanism that is yet to be defined (Scholz et al., 2005). A cytoskeleton-associated protein, *jwa*, which is a retinoic acid-responsive protein that like *hang*, can also regulate ethanol tolerance and cellular stress responses (Li et al., 2008), suggesting that ethanol tolerance and cellular stress responses

may share common molecular pathways (Kaun et al., 2012).

Krasavietz (*kra*), a translation initiation factor mediates behavioral sensitivity to ethanol intoxication. When assayed for both acute and chronic ethanol induced behaviors, *Kra* mutants show strong resistance to alcohol intoxication, reduced rapid and chronic tolerance, and reduced ethanol consumption (Berger et al., 2008; Devineni and Heberlein, 2009). Although only chronic tolerance has been reported to require protein synthesis (Berger et al., 2004), a pre-treatment with a protein synthesis inhibitor produced a strong decrease in sensitivity for ethanol intoxication, suggesting the existence of constitutively synthesized proteins necessary for acute ethanol responses (Kaun et al., 2012).

E. Genes affecting the actin cytoskeleton and its role in structural plasticity and alcohol addiction behaviors

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The actin cytoskeleton is one of the major components of the cellular scaffold that is essential for maintaining cell shape and size (Hotulainen and Hoogenraad, 2010). Actin dynamics support a myriad of processes ranging from cell migration, division and morphogenesis to intracellular protein trafficking (Cingolani and Goda, 2008). In developing neurons, the actin cytoskeleton has a

key role in axon guidance, neurite extension and branching, as well as synapse formation. Actin exists in two forms in the cell: globular (G) and filamentous (F) actin. G actin is the monomeric subunit that polymerize together to form an asymmetric two-stranded helical filament called F actin (Dillon and Goda, 2005). The assembly and disassembly of F-actin can be spontaneous, due to the weak non-covalent interactions of G-actin. However, at steady state and at a given cellular G-actin concentration, the difference in polymerization rates give rise to two ends: a net loss of actin monomers at the pointed (or minus) end and a net gain of F-actin at the barbed (or plus) end. This phenomenon is known as actin treadmilling, which helps in rapid turnover of G-actin while maintaining the length of F-actin at steady state (Dillon and Goda, 2005).

A variety of actin-binding proteins (ABPs) influence actin dynamics and the organization of the actin cytoskeleton. Capping proteins like tropomodulin and CapZ bind to filament ends and can modify filament turnover to affect their length (Lamprecht et al., 2004; Cingolani et al., 2008). Cross-linking proteins such as α-actinin, filamin, and spectrin can arrange F-actin into distinct arrays of networks. Other ABPs such as profilin promote F-actin polymerization while ADF/Cofilin depolymerizes F-actin. Cellular signaling pathways employ these ABPs to modify the synaptic architecture in response to changes in synaptic activity (Cingolani et al., 2008).

In mature neurons, actin is the most prominent cytoskeletal protein at synapses, present at both the pre- and postsynaptic terminals. The importance

and organization of actin at these terminals is evolutionarily conserved in *Drosophila* and mammals. From its subcellular organization, actin has been implicated in maintaining and regulating synaptic vesicle pools at the presynaptic terminals (Dillon et al., 2005). Synaptic vesicles are organized into at least two functionally distinct pools: the readily releasable pool (RRP), and the reserve pool (RP). The readily releasable pool consists of vesicles that are docked and primed for neurotransmitter release at the active zone of the presynaptic terminal. In larval *Drosophila* neuromuscular junction (NMJ) boutons, F-actin has been shown to be required for endocytosis and recruiting synaptic vesicles into the readily releasable pool. For instance, analysis of the *Drosophila* mutant strain N-ethylmaleimide sensitive Factor (NSF), a protein essential for the SNARE-complex disassembly and recycling that drive synaptic vesicle fusion, show a relationship between NSF activity and F-actin (Nunes et al., 2006). Additionally, loss of function *NSF2* mutants show decreased vesicle mobility and reduced F-actin levels at their NMJ boutons (Nunes et al., 2006). A deficit in either *Syntaxin 1A* (*Syx1A*), synapsin and *shibire* (*shi*), which encodes *Drosophila* dynamin, impairs ethanol tolerance in an experimental paradigm where synaptic vesicle release was selectively inhibited upon initiating the exposure to ethanol, but not after the sedation recovery period following the first exposure (Krishnan et al., 2011). It is difficult to dissect whether solely disrupting pre-synaptic mechanisms is sufficient to cause defects in ethanol tolerance or whether postsynaptic dysfunction arising from impairment in pre-synaptic mechanisms is the primary

cause. Whichever the case may be, the functions of *Synapsin* are many, thus it is feasible to speculate that hyper-tolerance to ethanol in *syn* mutants could stem from misregulation in neurotransmitter release, defects in neurite growth, synaptic formation and/or maturation. These phenotypes of Syn mutant could be linked to the actin cytoskeleton since the inability to prioritize the assembly of ready-to-release vesicles from those in the reserve pool phenocopies pharmacological inhibition of actin cytoskeletal dynamics (Cesca et al., 2010).

The RP are pools of synaptic vesicles that are released during intense stimulation. These pools are located at the center of the presynapse, where they are interlinked to each other by short F-actin filaments and synapsin (a presynaptic scaffolding protein) into clusters. Studies suggest that such a meshwork of filamentous actin, synapsin and vesicle creates a barrier to separate vesicle pools into two groups (Cingolani and Goda, 2008). This is evident from analyses of *Drosophila* larval NMJ boutons pretreated with Cytochalasin D, which inhibits polymerization of F-actin, leading to the elimination of the RP vesicles and reduced synaptic transmission evoked by high frequency stimulation (Kuromi and Kidokoro, 1998; Siechen et al., 2009).

In the postsynaptic terminals, actin is highly enriched in dendritic spines and at Post Synaptic Density (PSD) (Cingolani and Goda, 2008; Lamprecht and LeDoux, 2004). Dendritic spines are small protrusions formed on the main dendrite shaft, and receive inputs from excitatory presynaptic terminals such as glutamate and acetylcholine. Like mammals, *Drosophila* dendritic spines also

take on various shapes ranging from thin or stubby to mushroom or cuplike (Leiss et al., 2009). The likely role of actin in dendritic spines is to stabilize postsynaptic proteins and modulate spine head structure in response to postsynaptic signaling. Genetic experiments in *Drosophila* also indicate that actin rearrangements drive the formation and loss of dendritic spines. For instance, Drosophila FMRP (Fragile X mental retardation protein), a protein in humans known to cause Fragile X syndrome, is highly expressed in dendritic spines (Bushey and Cirelli, 2011). Loss of FMRP in both flies (*dfmr1*) and mammals, leads to a failure to remove immature synapses, while over expression of *dfmr1* in flies results in dendritic and axonal underbranching and loss of synapse differentiation (Bushey and Cirelli, 2011). Recent *dfmr1* studies suggest that profilin is also necessary for the development and morphogenesis of dendritic spines. Studies in profilin knockout mice also suggest that profilin is integral for stabilizing dendritic spines during synaptic plasticity and fear learning (Bushey and Cirelli, 2011; Reeve et al., 2005; Schenck et al., 2003).

As shown above, actin is integral to the formation, maintenance and plasticity of the synapse. Therefore, alterations in actin dynamics, particularly at the synapse, can have significant consequences for neuronal circuits and their underlying behaviors. Below, we will discuss how genetic studies in *Drosophila* show the importance of actin regulatory genes in learning and memory and the development of drug addiction.

Cell adhesion molecules

To establish and maintain structural organization at synapses, pre- and post-synaptic cells contact each other and the surrounding extracellular matrix (ECM) via cell adhesion molecules (CAMs). Many different classes of CAMs, including cadherins, protocadherins, neuroligins, neurexins, integrins, and immunoglobulin adhesion proteins are localized to synapses (Dityatev et al., 2008). CAMs regulate synaptic strength by recruiting scaffolding proteins, neurotransmitter receptors, and synaptic vesicles in response to coupling with like (homophilic) or other (heterophilic) cell adhesion receptors across the synaptic cleft (Brunton et al., 2004; Thalhammer and Cingolani, 2013). Neuroligins, synaptic cell adhesion molecules (SynCAMs) and integrins, are enriched at the center of the synapse (Mortillo et al., 2012), while others, like members of the cadherin family, are preferentially localized at the outer rims of pre-synaptic active zones and PSDs (Uchida et al., 1996).

Integrins are a class of transmembrane ECM receptors that function as αβ heterodimers and activate bidirectional-signaling cascades across the cell membrane (Grashoff et al., 2004). Integrins transduce information to the actin cytoskeleton via their direct and indirect interactions with ABPs. For instance, activation of the integrin receptor leads to the formation of cell adhesion complexes, consisting of many cytoplasmic proteins including talin, vinculin, paxillin, integrin-linked-kinase (ILK), parvins, and PINCH (particularly-interesting-cysteine- and histidine-rich protein) binding to the cytoplasmic tail of the β-integrin receptor subunit (Figure 3.1; Legate et al., 2006). Through these

complexes, integrin-linked ABPs like α-actinin (Honda et al., 1998; Legate et al., 2006; Pavalko and Burridge, 1991) and filamin (Loo et al., 1998; Sharma et al., 1995) attach to integrin and function as stable links for connecting the actin cytoskeleton to the ECM, thereby maintaining cell-ECM contacts (Figure 3.1).
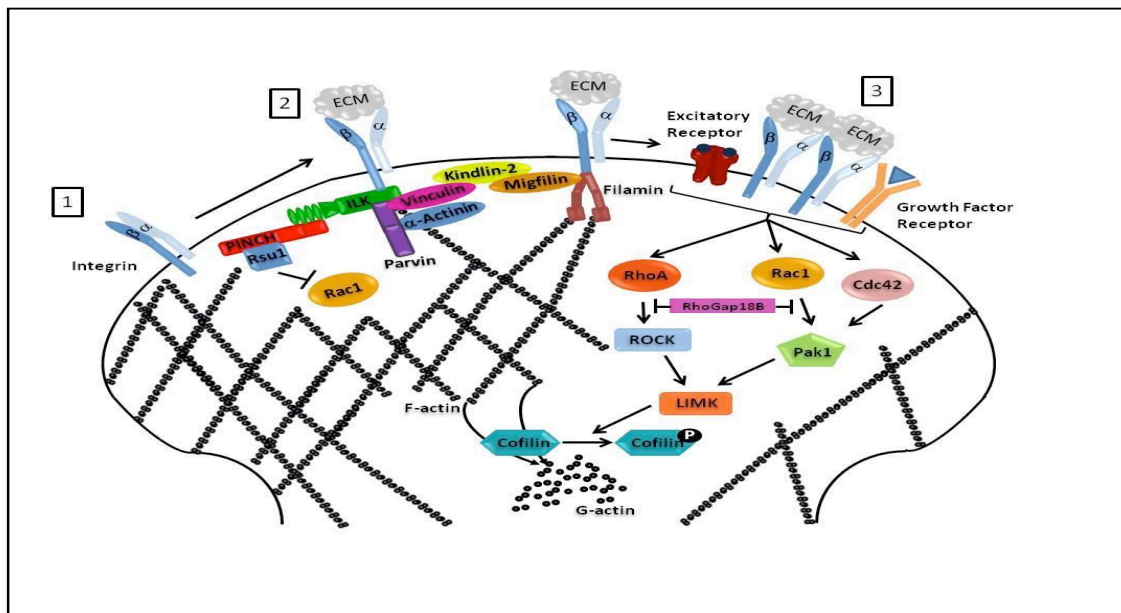
Figure 3.1. Model of the integrin receptor and Rho GTPases modulation of actin in the postsynaptic dendrite. Upon activation of by an ECM ligand, the integrin receptor (1) undergoes a conformational change leading to the formation of a cell adhesion complex at the cytoplasmic domain of the β-integrin subunit (2). Various proteins interact, and activate ABPs such as α-actinin and filamin to cross-link and connect actin filament bundles to the integrin receptor. Activation of the integrin receptor leads to the clustering of integrin receptors that can activate various growth factor receptors and affect various signaling pathways (3). Changes in the cellular actin cytoskeleton after integrin engagement are mediated through the Rho family of GTPases, Rac1, Cdc42 and Rho. Rac1 and Cdc42 phosphorylate Pak1 leading to LIMK-mediated phosphorylation, and inactivation of cofilin, which prevents depolymerization of F-actin to G-actin.

The importance of this link from integrin activation to F-actin filaments is highlighted by the finding that in rat hippocampal slices, LTP induction, and the concomitant increase in dendritic F-actin can be inhibited by anti-ß1 integrin antibody incubation in hippocampal slices (Kramar et al., 2006). In flies, integrins are highly expressed in a subpopulation of synaptic boutons at the CNS neuropil such as the mushroom bodies and a subset of synaptic boutons at the NMJs (Grotewiel et al., 1998; Rohrbough et al., 2000). It thus seems likely that loss of integrin signaling to the actin cytoskeleton would prevent proper regulation of synapse growth and sprouting. Indeed, this is the case, since loss of the α-integrin gene *volado (vol)* leads to a significant increase in synapse size and number, overgrowth of synaptic terminals, and increased dendritic branching in flies (Rohrbough et al., 2000). Additionally, *vol* mutant flies display abnormally elevated evoked transmission amplitudes and altered Ca^{2+} dependence of transmission at the NMJ, suggesting that integrin is required for normal short-term synaptic facilitation processes (Rohrbough et al., 2000). Similar to these fly studies, mammalian hippocampal culture studies support integrin's role in dendritic spine growth and plasticity. Using peptide inhibitors of integrin-ECM ligand interaction, the phenotypes observed include aberrant stability of LTP and actin-mediated structural remodeling, which were rescued by blocking N-Methyl-D-Aspartate receptor (NMDAR) function (Bahr et al., 1997; Shi and Ethell, 2006). Since NMDAR are required for the induction of LTP and structural plasticity, these data indicate a crucial role for integrin-mediated cell-ECM adhesion in

spine formation, as well as a role in neurotransmission-dependent morphological and physiological plasticity. Disruption of integrin signaling can therefore have profound effects on synapse plasticity and neural circuits that underlie certain behaviors.

A well-established behavior in *Drosophila* is aversive Pavlovian olfactory conditioning, where flies learn to avoid specific odors previously associated with electric shock (Quinn et al., 1974). Single session training in olfactory conditioning results in short-term (STM) and mid-term memory (MTM) retention (Tully and Quinn, 1985). Protein synthesis dependent long-term memory (LTM), on the other hand, is elicited only with repetitive spaced training and lasts for at least a week (Tully et al., 1994). The *vol* gene is required for proper formation of STM (Grotewiel et al., 1998). Compared to wild type*, vol* mutant flies showed memory deficits 3 minutes after training, suggesting that the formation, stability, or retrieval of STM is dependent on integrin function (Grotewiel et al., 1998). Another neural CAMs implicated in the formation of STM in *Drosophila* is Fasciclin II (the fly ortholog of NCAM2). Strains carrying mutations in *fasciclin II (fasII)* also show an STM defect (Cheng et al., 2001). Both *fasII and vol* are expressed preferentially in the mushroom bodies (MB), fly structures crucial for olfactory learning and memory (McGuire et al., 2003). Taken together, these studies support a model where integrin activation and signaling through ABPs enable the formation, and/or stability of activity- and experience-dependent structural changes in synapses essential for behavioral plasticity.

One of the strongest forms of behavioral plasticity in animals results from exposure to drugs of abuse, which highjack circuits normally engaged by natural rewards such as food and sex. When used repeatedly, drugs elicit molecular and structural changes at the synapse that promote continued drug craving, and this can supplant almost all other of the animal's behavioral goals (Hyman, 2005). These experience-, and drug-dependent reorganizations of neural circuitry require molecular mechanisms including CAM signaling. CAMs are also implicated in acute drug-induced behaviors such as sensitivity to ethanol-induced sedation. For example, the *fasII* gene is required for normal ethanol sensitivity in *Drosophila* (Cheng et al., 2001). Fly strains carrying mutations in *fasII,* when exposed to vaporized ethanol, take a shorter time than wild type flies to lose postural control, and then fall on their backs unable to right themselves (loss of righting or LOR), indicative of their ethanol-sensitivity. Similarly, flies carrying mutations in either the α-integrin receptor gene *scab* (*scb*) or β-integrin receptor gene *myospheroid* (*mys*) also cause increased ethanol sensitivity (Bhandari et al., 2009). A characteristic behavioral plasticity seen after acute ethanol exposure is the development of tolerance (Berger et al, 2008). Tolerance is defined as a decrease in the effect of a drug after repeated exposure, leading to a need for increased dosage to attain the same effect (Rodan and Rothenfluh, 2010). Tolerance is important in the development of drug dependence and addiction, and actin-dependent alterations in synapse structure are believed to play a major role. For instance, integrin's modulation of actin-mediated structural plasticity

plays a role in ethanol tolerance. *scb* and *mys* mutant flies, which are initially sensitive to ethanol, show increased tolerance to ethanol-induced loss of postural control 4 hours after the first ethanol exposure, when compared to wild type (Bhandari et al., 2009).

Activation of integrin can lead to the activation of various growth factor receptors such as epidermal growth factor (EGF), insulin receptor (InR), and vascular endothelial growth factor (VEGF), which also are implicated in learning and memory processes and the development of drug abuse (Brunton et al., 2004; Corl et al., 2009; McClure et al., 2011; see Figure 3.1). Although integrin receptors have many functions in various signaling pathways, dramatic changes in the cellular actin cytoskeleton after integrin engagement has been attributed to its signaling through the Rho family of GTPases.

Rho Family GTPases

As mentioned above, behavioral plasticity coincides with synaptic changes, including structural rearrangements. Postsynaptic dendritic spines commonly mature from filapodia, finger-like projections made up of bundled actin filaments, which establish the initial contact with axons (Korobova and Svitkina, 2010). Dendritic patches, where filapodia will form, contain a mixed network of linear and branched actin filaments, while the head of mature spines contains an actin meshwork similar to the one observed in lamellipodia, structures found in many dynamic cells (Halpain, 2000; Tada and Sheng, 2006; Sekino et al., 2007;

Hotulainen and Hoogenraad, 2010; Korobova and Svitkina, 2010). The major regulator of actin-dependent protrusions, morphogenesis, and structure is the Rho family of small GTPases, comprising Rho, Rac, and Cdc42. These GTPases act as molecular switches by cycling between an inactive GDP (guanosine diphosphate) form and an active GTP (guanosine triphosphate) form, which binds to, and activates downstream effectors, including ABPs (Heasman and Ridley, 2008). The proportions of GTP-, or GDP-binding is determined by three classes of regulatory proteins: guanine nucleotide exchange factors (GEFs) enhance the exchange of bound GDP for GTP; the GTPase activating proteins (GAPs) serve as negative regulators by increasing the rate of hydrolysis of bound GTP; and guanine nucleotide dissociation inhibitors (GDIs) inhibit both GTP exchange and the hydrolysis of GTP (Saneyoshi and Hayashi, 2012). Rho family GTPases play critical roles in the activity dependent formation and structural modification of dendrites in flies. For instance, loss of all three Rac genes, *Rac1*, *Rac2*, and *Mtl*, in *Drosophila* MB neurons result in a significant reduction in dendritic branching and length (Ng et al., 2002). Analysis of Cdc42 clones in vertical system (VS) neurons demonstrated a requirement for Cdc42 in regulating dendritic spine morphology, branching, and guidance (Scott et al., 2003). These phenotypes are similar to analyses of Cdc42 and Rac1 in cultured rodent hippocampal neurons, where dominant-negative expression of Cdc42 and Rac1 leads to a decrease in spine density (Impey et al., 2010; Irie and Yamaguchi, 2002; Tashiro et al., 2000), and expression of constitutive active Cdc42 and Rac1 cause an increase in spine

density (Impey et al., 2010; Tashiro et al., 2000). In contrast to Cdc42 and Rac1, the constitutive active form of RhoA decreases dendritic spine density and increases spine length, while a dominant negative form of RhoA increases spine density (Impey et al., 2010).

Within a single spine, the activities of RhoA and Cdc42 were analyzed in cultured slices of rat hippocampus during induction of LTP (Murakoshi et al., 2011). As the dendritic spine expands, the activity of both RhoA and Cdc42 were elevated for at least 30 minutes, depending on NMDAR and the Ca^{2+}/calmodulin-dependent kinase (CaMKII), which are both essential for LTP (Muller et al., 1988; Malinow et al., 1989). Activation of Cdc42 localized specifically to the stimulated spines, while RhoA diffused out from those stimulated spines (Murakoshi et al., 2011). Rac1 is also required for the formation and maintenance of LTP, since both mutant mice lacking the *Rac1* gene, as well as inhibition of Rac1 using pharmacological inhibitors affect spine structure and impair synaptic plasticity in the hippocampus, concomitant with hippocampus-dependent spatial learning defects (Haditsch et al., 2009; Rex et al., 2009). A particularly striking, and direct example of the importance of proper actin regulation in synaptic plasticity and behavioral learning was published recently by Huang and colleagues (2013). mTORC (target of rapamycin complex) is activated by numerous growth factor receptors. mTORC1 contains the protein Raptor, and is involved in cell growth and protein translation. Less well understood is mTORC2, which contains Rictor (rapamycin insensitive companion of mTOR). Mice with forebrain-specific Rictor

knock out do not show late phase LTP (L-LTP), and learn poorly in contextual fear conditioning (where mice normally learn to associate an environmental box with foot shocks, and therefore acquire box-induced freezing behavior). Similarly, flies lacking a functional *rictor* gene show normal STM, but no spaced training-induced LTM. Rictor knock out mice show decreased Rac1 activation, and a reduced F- to G-actin ratio, as well as fewer dendritic spines. Amazingly, these defects (fear memory, L-LTP, and F-/G-actin ratio) could be rescued by application of jasplakinolide to brain slices or direct injection into the brain. This marine sponge toxin promotes actin polymerization and in normal mice can also turn sub-threshold electro-physiological stimulation into L-LTP, as well as behavioral under-training into strong memories (Huang et al., 2013), illustrating the direct impact of actin polymerization on neural plasticity. Together, these studies suggest that (NMDA, integrin, and/or growth factor) receptor-mediated signaling pathways act via Rho family GTPases to regulate F-actin reorganization and spine morphology involved in synaptic, and behavioral plasticity, as well as learning and memory.

Acquired memory that is not reinforced by repetitive learning is vulnerable to being erased or forgotten (Shuai et al., 2010). A recent report showed that Rac1 contributes to both passive memory decay and forgetting in *Drosophila* (Shuai et al., 2010). Over-expression of a dominant negative form of *Drosophila* Rac1, Rac1^{DN} in neurons led to normal memory acquisition in the first 30 minutes after training, but significantly slowed memory decay at later time points from 2

hours to 24 hours (Shuai et al., 2010). This delay in memory decay is independent of protein synthesis and therefore does not resemble LTM. The Rac1^{DN} expressing flies also did not forget previously trained odor even when perturbed 1.5 hours later, by training with a new aversive odor (interference-learning paradigm). Conversely, over-expression of constitutively active form, Rac1^{CA}, accelerated memory decay. In wild-type flies, Rac1 activation also correlated with memory decay, suggesting that memory can be bi-directionally regulated through the manipulation of Rac1 (Shuai et al., 2010). Interestingly, in conjunction with previous studies discussed, these experiments suggest that Rac1 activation has a critical role in both the acquisition, as well as in the active erasing/forgetting of memories. It also highlights the importance of controlling not only synapse strengthening, but also weakening and elimination in the normal context of daily experiences.

Rho GTPases and their effectors also play a role in ethanol-induced behaviors in *Drosophila.* Neuronal expression of activated Rac1 GTPase leads to ethanol-resistance, the same phenotype flies carrying mutations in RhoGAP18B, a protein that inactivates Rho family GTPases such as Rac1 and Rho1 (fly ortholog of RhoA; Figure 3.1), display (Rothenfluh et al., 2006). Conversely, flies with decreased Rac1 function are sensitive to ethanol-induced sedation (Rothenfluh et al., 2006). Flies lacking Arf6, a member of the Arf family of GTPases that function in membrane trafficking and actin organization, are also sensitive to ethanol-induced sedation (Peru y Colón de Portugal et al., 2012).

Rac1 functionally connects to Arf6 via the BAR domain protein Arfaptin, which can directly bind to Rac1 as well as Arf6. Flies lacking Arfaptin are also ethanol-sensitive (Peru y Colón de Portugal et al., 2012) and show synaptic undergrowth at the *Drosophila* NMJ (Chang et al., 2013). These studies in flies and mammals continue to confirm that actin plays a role in the development of drug abuse.

Above mutants, with their altered synaptic structures, could predispose the animals to react differently to ethanol exposures. But are there acute effects of ethanol on the actin cytoskeleton? It has been known for a while that exposure in cell culture leads to profound changes in cell shape. For instance, chronic exposure of primary astrocytes to ethanol (30 mM for 7 days) alters the actin cytoskeleton, with a marked increase in F-actin near the plasma membrane (Tomas et al., 2003). The ethanol-induced changes in actin are likely due to an ethanol-induced decrease in Rho family GTPase activity, especially RhoA, since treatment with lysophosphatic acid (LPA), an activator of RhoA (Tomas et al., 2003), or transfection with activated RhoA (Guasch et al., 2003) blocks the ethanol-induced effects. Conversely, astrocyte cultures treated acutely with ethanol (100mM for 10 minutes) have reduced stress fibers, which are rich in F-actin (Allansson et al., 2001; Guasch et al., 2003), suggesting a rapid change in RhoA activity. One potential mechanism for reduced RhoA activity is via upregulation of p190 RhoGAP, converting active RhoA-GTP to inactive RhoA-GDP. Chronic alcohol exposure increases p190 RhoGAP activity and redistributes it to the plasma membrane (Selva and Egea, 2011), but the precise

mechanism(s) remains unclear. Nevertheless, these data suggest that acute ethanol has a negative effect on F-actin stability, and that the observed long-term increases in plasma membrane actin filaments may be a compensatory reaction to prolonged ethanol exposure (Rothenfluh and Cowan, 2013).

Insights into the acute effects of ethanol on actin and neuronal function have come from a number of studies. Popp and Dertien (2008), reported that a brief 30 second pre-exposure of cultured cerebellar granule cells to ethanol potentiated subsequent direct NMDAR inhibition by ethanol, even when the pretreatment was applied intracellularly. Phalloidin, an F-actin stabilizer, prevented this potentiation, while latrunculin A (latA), an actin depolymerizer, mimicked the effect (Popp and Dertien, 2008). These findings suggest that acute ethanol leads to F-actin instability, and causes a decrease in NMDAR current, which was also found in cerebellar granule cell slices (Offenhauser et al., 2006). Knocking out EGF receptor pathway substrate 8 (EPS8) in mice, an actin capping protein, suppressed both ethanol-induced NMDAR current rundown and F-actin instability. Behaviorally, *EPS8* knockout mice were resistant to ethanol-induced LOR and showed increased alcohol consumption in a 2-bottle choice assay (Offenhauser et al., 2006). EPS8 localizes to postsynaptic densities in cerebellar granule neurons, and can activate the small GTPase Rac1 (Offenhauser et al., 2006). Similar to mammals, loss of the fly ortholog of EPS8, called *arouser,* also affects ethanol-induced LOR, and it also affects synapse

number (Eddison et al., 2011), suggesting another link between actin and neuronal structure and function.

Aside from alcohol, members of the Rho family of GTPases are also linked to other drugs of abuse, such as nicotine and cocaine, in both flies and mammals. Loss of RhoGAP18B makes flies resistant to both nicotine and cocaine-induced LOR, for example (Rothenfluh et al., 2006). Recently, Dietz et al. (2012) showed that the small GTPase Rac1 affects cocaine reward in the nucleus accumbens (NAc). They found that acute intraperitoneal injections of cocaine in mice led to transient reduction in active Rac1 and expression of dominant-negative Rac1 enhanced both dendritic spine numbers as well as cocaine-induced place preference (where mice positively associate a box with cocaine; Dietz et al., 2012). Kalirin, a guanine nucleotide exchange factor (GEF) for the Rho-family, activates the small GTPase Rac1, and is highly enriched in the postsynaptic density of rat cerebral cortex (Kiraly et al., 2011a; Kiraly et al., 2010; Penzes et al., 2000). Knockout mice of Kalirin-7 show reduced conditioned place preference (a paradigm where cocaine in used as a drug reinforcer of place memory) (Kiraly et al., 2011b; Rothenfluh and Cowan, 2013). Kalirin knockout mice also show reduced levels of the NMDAR subunit NR2B (Kiraly et al., 2011a; Rothenfluh and Cowan, 2013). These studies suggest that the same molecules that are involved in learning and memory also participate in drug-induced plasticity, even though in the case of Rac1, they seem to have opposite effects, with dominant-negative Rac1 enhancing cocaine-induced plasticity, while normal Rac1 activity is required

for L-LTP and fear conditioning (Huang et al., 2013). Then again, Rac1 activity in flies is also required for forgetting (Shuai et al., 2010), and therefore the acute decrease in cocaine-induced Rac1 might increase place-preference by reducing memory decay, a possibility not explored in the experiments by Dietz and colleagues (2013). These studies on Rac1 do highlight both the importance of this actin-regulating small GTPase, as well as the requirement for fine-tuned Rac1 regulation for proper neuronal and behavioral plasticity.

Effect of ABPs and other Actin Regulatory Genes on *Drosophila* Behavior

One of the downstream effectors of Rac1 is the actin-severing protein cofilin. It is inactivated by phosphorylation, which can be triggered by Rho family GTPases. GTP-bound Rac1 and Cdc42 activate p21-activated kinase (PAK), which in turn phosphorylates and activates Lin11/lsl-1/Mec3 kinase (LIMK), which in turn inactivates cofilin. RhoA can activate LIMK via activation of Rho-associated kinase ROCK (Schubert and Dotti, 2007). Within spines, cofilin is thought to be critically involved in the structural changes triggered by experiences leading to stable modifications in synaptic responses (Fedulov et al., 2007; Figure 3.1). Cocaine-conditioned place preference is suppressed by photo-activated Rac1, which is mediated by cofilin inactivation. Photo-activation of Rac1 causes phosphorylation of cofilin, and expression of dominant-negative (pseudo-phosphorylated) cofilin recapitulates the behavioral suppression seen with Rac1 (Dietz et al., 2012). Furthermore, cofilin also functions downstream of Rac1 to

regulate memory decay and forgetting, since neuronal expression of the constitutively active form of cofilin enhanced 3 hour memory performance similar to Rac1 inhibition (Shuai et al., 2010). Cofilin is thus a direct actin modulator critical for synaptic and behavioral plasticity.

Behavioral and neuronal changes are also affected by actin capping proteins, as illustrated by the β-adducin knockout mouse, which has defects in hippocampal LTP, as well as deficits in several learning assays (Rabenstein et al., 2005). Hts, the fly ortholog of this actin capping protein found at pre-synaptic terminals has not been shown to affect learning and memory, but loss of *hts* results in a dramatic increase in the number of synaptic retractions, as well as a generalized overgrowth of large-diameter glutamatergic type Ib boutons at the larval NMJ (Pielage et al., 2011; Stevens and Littleton, 2011). As mentioned earlier, the actin capping protein EPS8 is involved in ethanol responses in both flies and mice, and a number of other ABPs affect both drug-induced behaviors, and learning and memory. For example, filamin, an actin cross-linking protein previously discussed as binding to the β-subunit of integrin (Figure 3.1), is necessary for learning and memory, and for drug-induced behaviors since loss of filamin (*cheerio* mutants) causes sensitivity to ethanol-induced sedation, and deficits in olfactory LTM formation (Berger et al., 2008; Bolduc et al., 2010). Formin3*,* an ABP that nucleates the formation of unbranched actin filaments also regulates ethanol sensitivity, tolerance, and LTM formation in flies (Berger et al., 2008). Lastly, the synaptic vesicle, and actin binding protein synapsin has

already been discussed as affecting fly ethanol tolerance, courtship and olfactory conditioning (see above; Godenschwege et al., 2004)

All these studies suggest that common neurobiological mechanisms contribute to the development of synaptic and dendritic spine plasticity. These mechanisms are required for both drug addiction and for learning and memory. Indeed, many fly mutants isolated by their behavioral defects in associative learning and memory also show defects in ethanol-induced behaviors such as tolerance, or acute ethanol sensitivity (Berger et al., 2008). This is not surprising, however, since the current view is that drugs of abuse highjack natural reward centers in the CNS. Exposure artificially reinforces the drug-associated experiences, thereby causing long-lasting changes in the brain that underlie the behavioral abnormalities associated with drug addiction (Hyman, 2005). Common experiences of environmental stimuli normally induce memory formation and stable changes in the brain as well. Drug addiction can thus be viewed as a disease of pathological learning (Nestler, 2002), utilizing existing plasticity mechanisms, including actin-mediated structural alterations.

Other cytoskeletal elements such as microtubules have been implicated in ethanol-induced behaviors. For instance, the *Drosophila* allele that functions through Kinase Par-1, *thousand and one* (*tao*), regulates microtubule dynamics and its microtubule-associated protein Tau, in order to mediate ethanol-induced hyperactivity (King et al., 2011; Matenia and Mandelkow, 2009).

F. Conclusion

Here, I have highlighted that a large number of genes affect synaptic plasticity and alcohol-induced behaviors, emphasizing the model organism D. melanogaster. In addition to stressing the link between actin dynamics, structural plasticity, I hope to have reiterated the usefulness of this genetically tractable model system – both as a tool to find novel genes (See chapter 2) as well as a means to test the in vivo relevance of molecularly characterized proteins and signaling cascades in AUDs.

Studies mentioned above show that Rho-family GTPases play a role in alcohol responses. However, the upstream signaling pathways modulating their effects on actin cytoskeletal dynamics are not fully understood. Using behavioral methods described in chapter 2, I show in chapter 5 that the integrin/Rsu1/Rac1-signaling pathway is an important modulator of drug-induced reward/behavioral plasticity, including ethanol consumption in flies and humans. Before getting into the results, I will first give an overview of the function and anatomy of fly adult brain and how they affect alcohol-induced behaviors.

**CHAPTER 4: Overview of brain anatomy and dopamine circuits in
*Drosophila melanogaster***

Neural circuits in the brain are the substrates for sensory processing and integration, which ultimately lead to animal behavior. These behaviors and the changes that result from experience are dependent on which neurons communicate with each other and how these (mostly) synaptic communications change with experience. In chapter 5, I discuss a gene called Rsu1 that have differential requirements in different anatomical structures to affect alcohol-induced behaviors ranging from naïve sedation to alcohol consumption/preference. These results led to the question of deciphering what neural circuits are involved within these fly brain structures to affect initial drinking and also compulsive/chronic alcohol consumption in flies. In this chapter, I will give an overview of the fly-brain anatomy involved in ethanol-induced behaviors and how distinct dopaminergic neurons innervate different fly brain structures to induce a certain behavior. This will provide a background for the upcoming chapters (9-10).

Anatomy of the fruit fly's central nervous system

Apart from the genetic conservation between *Drosophila* and humans, similarities in their brains are also evident. Although the Drosophila CNS is anatomically distinct and clearly of lesser complexity than the mammalian CNS, evidence for some deep evolutionary homology regarding the ancestry and function of whole brain regions continues to emerge (e.g., Strausfeld and Hirth,

2013).

The vinegar fly nervous system consists of a brain, segmented nerve cord, and peripheral nervous system. The adult fly brain has various segregated nuclei dedicated to taste, vision, olfaction, learning and memory, much like one would find in vertebrate brains. These regions are defined as neuropils separated by glial compartments (Younossi-Hartenstein et al., 2003). The major brain centers of the fly are the antennal lobes, the central complex, the descending neurons, median bundle, mushroom bodies, optic lobes, suboesophageal ganglion, and the pars intercerebralis. As my interest lies in brain regions involved in alcohol-induced behaviors, I will only be discussing brain neuropils such as the central complex and the mushroom bodies because of i) their roles in learning and ii) their roles in alcohol-induced behaviors such as tolerance, locomotion, and dopamine regulated behaviors.

The Mushroom Bodies

The Mushroom bodies (MB, Figure 4.1) are the most prominent structure in the adult fly brain studied for its role in in associative olfactory conditioning, learning and memory processes, sleep, and its role in addiction (Mcbride et al., 1999; Mcbride et al., 2005; Busto et al., 2010; Shuai et al. 2010; Hendricks et al., 2010, Kaun et al., 2012). The MBs are lobed neuropils that comprise long and approximately parallel axons originating from clusters of minute basophilic cells called Kenyon cells (KCs) located dorsally in the most anterior neuromere of the central nervous system (Aso et al., 2014). MB structures are found conserved in

annelids and in all arthropod groups except crustaceans. Insect MBs usually have two or more sets of lobes arising from the pedunculus at the front of the brain with *Drosophila melanogaster* having both a vertical lobe and a medial lobe assemblage (Strausfeld et al., 1993, Figure 4.1). The medial and vertical lobes are each divided into two parallel components called, respectively, α, α_1, β and γ. About 2000 KCs axons form the medial and vertical lobes while their dendrites form the mushroom body calyces. Major Inputs into the MB arise from the antennal lobes of the fly brain, which regulates odor sensing in flies. Dopaminergic (DA) and octopaminergic neurons (Aso et al., 2014) also innervate the MB and aid regulation of appetitive and aversive valences (Schwaerzel et al., 2003). The MB KCs form synapses with a relatively small number of about 34 MB output neurons (MBONs). The MBONs have dendrites in the MB lobes and project axons to neuropils outside of the MB structure. MBONs that use the same neurotransmitter extend dendrites to adjacent regions of the lobes; cholinergic MBONs in the vertical (α and α') lobes, glutamatergic MBONs in the medial (β, β', and γ) lobes, and GABAergic MBONs in an area of the lobes at the intersection between these two regions (Aso et al., 2014).

Comparisons between the vertebrate hippocampus and invertebrate MBs have been proposed, since both play roles in similar types of learning and memory, such as associative memory, and context dependent sensory filtering, (Aso et al., 2014; Mao et al., 2009). In addition to these similar functions, drugs of abuse modulate dopamine signaling in these structures to affect odor-associated

alcohol preference (Kaun et al., 2012), and the development of alcohol preference (experience-dependent alcohol preference, EDAP) in flies (See Chapter 5, and 8).
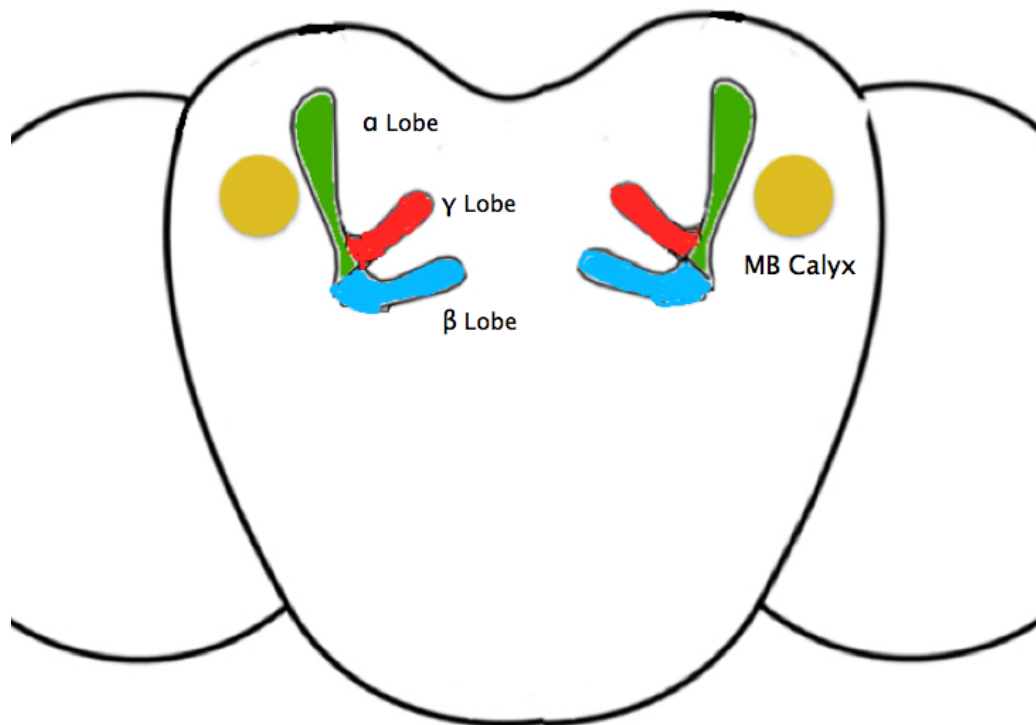
Figure 4.1: Schematic showing Adult fly brain image of the Mushroom body structures. The anterior section of the adult fly brain shows the α (green), β (blue) and γ (red) lobe assemblage of the MB structure. Posterior section of the adult fly brain shows the mushroom body calyx (yellow), which contains the Kenyon cells. Kenyon cells of the calyx send projections to anterior lobes of the MB. Adapted from Kong et al., 2010.

The Central Complex

The central complex serves as an integration center for diverse motor, sensory modalities (i.e. vision and taste), learning, and memory activities in insects (Wolff et al., 2014; Kong et al., 2012; Ofstad et al., 2011). It is also involved in coordinating locomotor behavior, including flight and various aspects of walking in flies (Ofstad et al., 2011). From anterior to posterior projection of the drosophila brain, the central body complex (CBC) comprises the ellipsoid body, the superior arch and fan shaped body above the paired noduli, and the protocerebral-bridge. According to Strausfeld, structures in the CBC show homology in terms of cytology, brain innervations, function, and behavioral outcomes to structures of the vertebrate basal ganglia, and are discussed below.

Fan-Shaped Body

The fan-shaped body (FSB) is a structure of 6-8 horizontal layers and 16 vertical slices (sometimes called staves, columns or segments), 8 per hemisphere numbered from medial to lateral - arranged in 4 closely associated pairs. It contains arborizations of efferent, intrinsic, and afferent neurons. As the name implies, it is shaped like a fan (Figure 4.2). The FSB in addition to the **protocerebral-bridge** (a rod like neuropil composed of a chain of 16 glomeruli and provide innervation to the FSB, Figure 4.2) have similarities in organization and function to the striatum of the vertebrate brain (Strausfeld et al., 2013).

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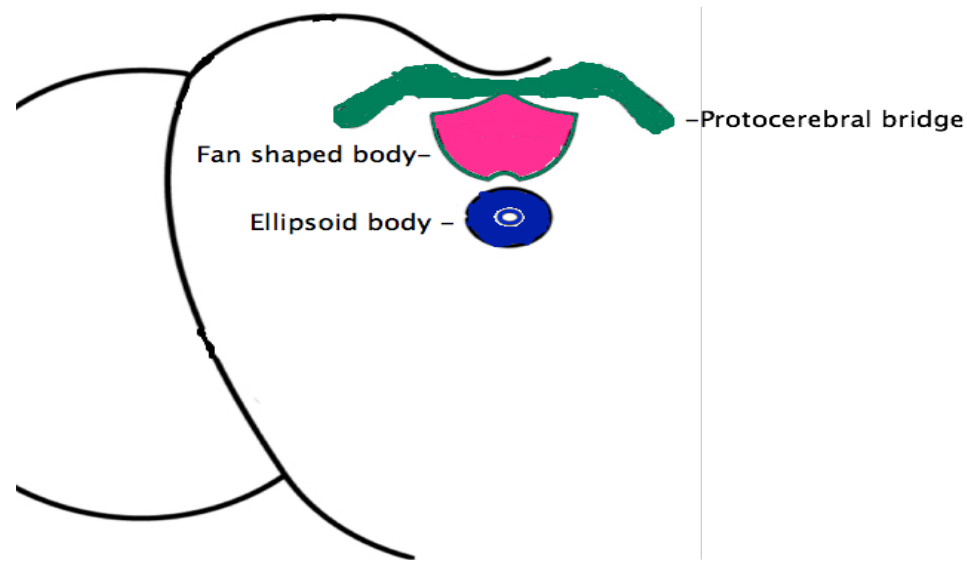
Figure 4.2: Schematic showing adult fly brain image of structures found in the central complex. The central complex of the adult fly brain contains different neural structure that includes the Fan shaped body (red), the ellipsoid body (blue) and the protocerebral bridge (green). Adapted from Kong et al., 2010.

Ellipsoid Body

The ellipsoid body (EB, Figure 4.2) structure is required for visual pattern place memory (Ofstad et al., 2011) and diverse motor functions that includes locomotor hyperactivity. It is not surprising that the EB is thus required for drug-induced hyperactivity (Kong et al., 2010; Lebestky et al., 2009). The EB is an almost circular neuropil lying anterior to the fan-shaped body. It consists of ring-like terminals of neurons arising in the lateral protocerebrum (Wolff et al., 2014). The vertebrate pallidum and the ellipsoid body also show similar circuit organizations.

In vertebrates, the globus pallidus (GP) structure contains GABAergic neurons that connect to the subthalamic nucleus and the thalamus, with reciprocal glutamatergic connections from the subthalamic nucleus back to the GP (Graybiel, 2000; Kandel et al., 2000). Inhibitory outputs from the globus pallidus serve to select specific motor actions by suppressing inhibition of their activating circuits. Inhibitory GABAergic neurons in the fruit fly provides dense networks in the fan-shaped body, ellipsoid body, and two satellite centers (**noduli**), which extend their axons laterally from the central complex flanking the lateral accessory lobe neuropils (Wolff et al., 2014; Strausfeld et al., 2013).

These anatomical structures described above are innervated by distinct dopaminergic neurons and are implicated to play a role in alcohol-induced behaviors. In the next section, I will described the functional and anatomical organization of dopaminergic neurons in the fruit fly central nervous system

Functions of Dopamine in fruit flies

Dopamine in fruit flies modulates various behaviors such as locomotion (Pendleton et al., 2002), response to sugar (Marella et al., 2012), sleep and arousal (Liu et al., 2014, Ueno et al., 2012; Andretic et al., 2005; Foltenyi et al., 2007; Kume et al., 2005), aggressive behavior (Alekseyenko et al., 2013), courtship behavior (Liu et al., 2008; Neckameyer, 1998), inhibition of startle-induced hyperexcitability (Friggi-Grelin et al., 2003), saliency-based decision making (Zhang et al., 2007), and associative learning, the latter often measured by olfactory classical conditioning using an odor as conditioned stimulus (CS).

Functions in appetitive olfaction (Wang et al., 2013), aversive olfaction (Aso et al., 2012), reward signaling (Burke et al., 2012, Liu et al., 2012), and learning (Berry et al., 2012) have also been associated with individual dopaminergic neurons and receptor subtypes. These studies disprove previous studies suggesting that inhibition of neurotransmitter release from dopaminergic (DA) neurons impairs the formation of aversive, but not appetitive, olfactory memory (Schwaerzel et al., 2003; Tempel et al., 1984). PAM neurons where recently identified and shown to be required for appetitive rewards in flies, suggesting the dopamine is required for modulating both aversive and appetitive rewards in flies.

Abnormal functional states of the DA system underlie some behavioral disorders in humans. Depletion of dopamine or its receptors in the nigrostriatal pathway can result in a range of pathologies, most of which refer to dysfunction of inhibitory or activation control of motor behaviors. For instance, progressive loss of dopaminergic neurons in the substantia nigra causes loss of the nigrostriatal pathway, which in turn results in Parkinson's disease characterized by a progressive increase in the brain's inability to suppress motor actions, leading to rigidity, bradykinesia, and nonmotor symptoms including sleep and mood disorders (Graybiel, 2000). Similar in flies, dopaminergic neurons and D1 receptor activation in the central complex play crucial roles in behavioral action selection and maintenance. Age-related degeneration of dopaminergic neuron clusters also leads to Parkinsonism in Drosophila as characterized by severely impaired motor behavior (Hirth, 2010). Additionally, specific depletion of

dopamine in flies results in reduced activity and locomotor deficits, extended sleep time, and defects in aversive olfactory-memory formation, suggesting that arousal and choice require normal dopamine levels (Riemensperger et al., 2011).

Like mammals, dopamine in flies plays a critical role in the locomotor hyperactivating effects of ethanol. Silencing dopaminergic neurons pharmacologically or genetically in flies leads to a reduction in ethanol-induced locomotion (Bainton et al., 2000; Kong et al., 2010). Furthermore, expression of the D1-like receptor (Dop1R) in the ellipsoid body is required for locomotor activity elicited by ethanol exposure (Kong et al., 2010). Perturbation of dopamine signaling in flies also regulates reward learning in flies. Silencing dopamine neurons blocks odor preference in an ethanol-reinforced odor conditioning paradigm (Kaun et al., 2011) suggesting dopamine's role in regulating reward circuits. Taken together, these homologies between the functions of dopamine in flies and human suggest that the fly is a very good model organism for studying different forms of dopamine-dependent pathologies and can therefore be used to try to identify circuits affecting alcohol drinking and reward in flies.

Anatomical and functional organization of dopaminergic neurons in the fly central nervous system

Dopaminergic (DA) neuron specification in fruit flies occurs near the end of embryonic development (Neckameyer and White, 1993). In the larval ventral ganglion, there are dorsal segmented pairs and unpaired cell bodies whose axons terminate ventrally into wide arborizations along the anterior-posterior

tracts. In the brain, there are also paired and unpaired cell bodies that send projections to almost every neuropil. Anatomy of the larval dopamine system is highly stereotyped with almost no variation between animals. About 90 DA neurons are found in the larval nervous system (Budnik and White, 1986, Selcho et al., 2009) with 30 cell bodies are seen in the brain and 60 found in the ventral nerve cord. Generally the cell bodies are located peripherally with their axons projecting into brain neuropils and fascicles. All four regions of the protocerebrum and the sub esophageal ganglion receive extensive dopaminergic innervations.

Variable numbers of dopaminergic cells have been found in studied adult insect brains (Sykes et al., 2004). DA neurons typically form about 8 paired clusters named based on their locations (Figure 4.3): paired posterior lateral 1 and 2 (PPL1 and PPL2), paired posterior medial 1 and 2 which are typically grouped together (PPM1/2), PPM3, paired anterior lateral (PAL) and paired anterior medial (PAM) (White et al., 2010). The anterior cells and the PPM1/2 cells innervate the protocerebrum. Different clusters of dopamine-containing neurons identified at the level of single nerve cells have stereotypic anatomical and location-specific projection patterns with DA clusters highly innervating different sections of the MBs (Mao and Davis, 2009).
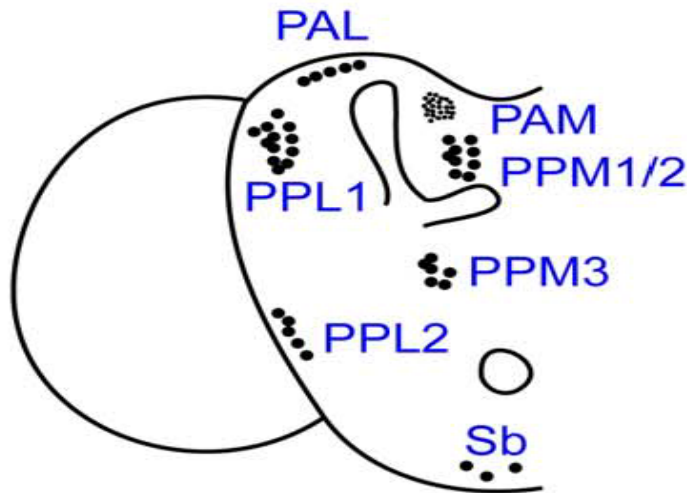
Figure 4.3: Dopaminergic neuron cell cluster positions in one hemisphere of the adult brain. Schematic showing 7 distinct dopaminergic cell clusters in the fly brain. Adapted from Kong et al., 2010.

Very little is known about the neurons and circuits mediating alcohol-induced behaviors. Early approaches were hampered by the lack of tools, i.e. Gal4 lines expressing in small, specific regions of the brain that would allow gene, or neuron manipulation of these brain regions. But with the completed development of over 5000 new, more specific Gal4 lines, and combinatorial approaches that allow for the further refinement of these expression patterns, anatomical studies will become increasingly feasible (See chapter 8 and 9). An understanding of the neurons and circuits, mediating alcohol-induced behaviors will have three benefits. First, scientists will learn about the basic, neural architecture that mediate behaviors, such as motor activation, or the development of preference. Second, researchers will be able to use these Gal4

lines as anatomical tools to very specifically alter gene function in only the neurons that are relevant to the behavior. This way one can circumvent other potentially deleterious effects these genes might have in other brain regions, and we will be able to better understand the genetics of these behaviors. Lastly, one can ask whether there are brain regions in the fly that are functioning analogously to the mammalian ventral tegmental area, or the nucleus accumbens. These two structures are intimately involved in the development of addiction, and we will be able to test whether the amazing functional conservation that is observed between flies and mammals regarding the molecular and genetic mechanisms involved in alcohol responses also extends to the structure and organization of the brain. Since dopamine plays a key role in the development of addiction, in chapter 8, my data show that distinct dopaminergic circuits are required for the aversive and appetitive properties of alcohol in flies just like in mammals. In the next 2 chapters, I will first show that genes regulating actin are required in different structures to affect behaviors ranging from alcohol sensitivity to alcohol preference

CHAPTER 5: Ras Suppressor 1 regulates ethanol consumption in Drosophila and Humans

*This chapter has been accepted for publication at PNAS in 2015. I collected data that led to figures. Human Data in this chapter was obtained in collaboration with the Schumann Lab and members of the IMAGEN Consortium. I co-wrote the manuscript with Dr. Rothenfluh and Dr. Rodan, and the Schumann Lab edited/approved the final version for publication manuscript.

Introduction

Alcohol consumption has a worldwide prevalence of 42% (World Health Organization, 2004) and alcohol is the third most serious risk factor for health-loss worldwide (Lim et al., 2012). The genetic contribution to the development of alcohol use disorders (AUDs) has been estimated at 40-60%, based on family, adoption and twin studies (Gelernter and Kranzler, 2009; Dick et al., 2006). Even though several studies in humans and model organisms have described genes and molecular pathways involved in alcohol responses (Schumann et al., 2006; Joslyn et al., 2011), our molecular understanding of how AUDs develop is still incomplete.

The vinegar fly, *Drosophila melanogaster*, is a genetically tractable organism used to model addiction-relevant ethanol-induced behaviors (Rodan and Rothenfluh, 2010; Kaun et al., 2012). When exposed to ethanol vapor, flies display biphasic behaviors similar to those elicited in humans. Low ethanol doses induce a state of disinhibition and increased locomotor activity, while higher doses lead to loss of postural control and sedation (Lee et al., 2008; Wolf et al., 2002). Flies also display addiction-like behaviors similar to mammals. In an ethanol consumption and preference assay (Ja et al., 2007), for example, flies gradually acquire alcohol

preference and will overcome an aversive stimulus in order to consume alcohol (Devineni et al., 2009). In addition to the similarities that mammals and flies display in their behavioral responses to ethanol, numerous genes and signaling pathways affect alcohol-induced behaviors across organisms. *In vitro* and *in vivo* studies in *Drosophila* and mammals have revealed a link between alcohol and the actin cytoskeleton (Rothenfluh and Cowan, 2012). When cultured primary mouse neurons are exposed to ethanol, there is a gradual decay in filamentous actin that correlates with decreased NMDA receptor current (Offenhauser et al., 2006). Mice with a genetic knockout of the actin-capping protein EPS8, which display reduced decay of both filamentous actin and NMDA receptor current in the presence of acute ethanol, show increased alcohol preference (Offenhauser et al., 2006). Flies with mutations in the *arouser* gene, encoding an EPS8 homolog, also show an ethanol-sensitivity phenotype (Eddison et al., 2011).

A major regulator of actin cytoskeleton dynamics is the Rho-family of small GTPases including Rho, Rac, and Cdc42, and mutations in these genes affect alcohol-induced behaviors (Rothenfluh and Cowan, 2012). Adult loss of Rac1 activity, for example, leads to enhanced sensitivity to alcohol-induced sedation, while loss of the Rac1 down-regulator RhoGAP18B causes reduced sensitivity (Rothenfluh et al., 2006). Although these studies have shown that Rho-family GTPases play a role in alcohol responses, the upstream signaling pathways modulating their effects on actin cytoskeletal dynamics are not understood.

Here, we describe the identification and characterization of mutations in the

icarus (*ics*) gene, encoding Ras suppressor 1 (Rsu1), which exhibit reduced sensitivity to ethanol- induced sedation. Our experiments reveal that *ics* mediates normal behavioral responses to ethanol in the adult nervous system by regulating actin dynamics downstream of integrin, and upstream of the Rac1 GTPase. While wild-type flies gradually acquire ethanol consumption preference over several days, flies completely lacking Rsu1 show heightened naïve preference that does not increase further over the time of the assay. Conversely, flies lacking Rsu1 only in the mushroom bodies show no naïve preference and also fail to acquire preference over time, suggesting that distinct neural circuits mediate naïve and acquired ethanol preference. In humans, *RSU1* was associated with frequency of lifetime drinking in an adolescent sample and the amount of alcohol consumed in both an adolescent sample and an independent adult replication sample. In adolescents, *RSU1* was also associated with altered fMRI activation in the ventral striatum during reward anticipation. Our findings thus highlight Rsu1, and the integrin/Rsu1/Rac1 signaling pathway as an important modulator of reward-related phenotypes, including ethanol consumption across phyla.

Results

ics Mutants Display Reduced Sensitivity to Ethanol-Induced Sedation

To identify genes involved in ethanol-induced behaviors in *Drosophila*, we screened a collection of strains carrying random insertions of a transposable P element. We isolated one mutant that displayed reduced sensitivity to ethanol-induced sedation when compared to controls (Figure 5.1*A,* 5.1*C*). DNA sequencing

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analysis revealed that the Gal4-containing P element in this line is inserted in the *icarus (ics)* gene, and we thus labeled it *ics*^{G4}. The *ics* gene had been previously identified because of its wing blister phenotype (Kadrmas et al., 2004), and *ics*^{G4} mutant flies also exhibited wing blisters. The original mutant, *ics*^{BG}, carrying a P element insertion at the 3' end of *ics* exon 3 (Fig. 5.1*F*), showed reduced sensitivity to ethanol-induced sedation similar to that of *ics*^{G4} (Fig. 5.1*C*). Heterozygous *ics* flies showed no phenotype, and were used as controls in some experiments below. To confirm that the transposon inserted in *icsG4* was indeed responsible for the *ics*^{G4} ethanol phenotype, we mobilized the *ics*^{G4} P element by supplying transposase enzyme. Precise excision (*ics*^{x23}) of the P element reverted the mutant phenotype to wild-type, while imprecise excision of the P- element (*ics*^{x5}, resulting in a deletion of 1353 bp, Fig. 5.1F) showed the *ics* mutant phenotype (SI Appendix, Fig. 5.1*C*). Expression of the Rsu1 protein was absent in *ics* mutants (*ics*^{G4}, *ics*^{x5}) and normal in the precise excision *ics*^{x23} (Fig. 5.1*D*). The reduced ethanol sensitivity in *ics* mutants was not due to altered pharmacokinetics, as ethanol absorption and metabolism were normal in *icsG4, ics*^{BG}, *and ics*^{x5} flies when compared to controls (Fig. 5.1*G*). Flies carrying mutations in *ics* also showed normal locomotion (assessed by startle-induced phototaxis and negative geotaxis, and by spontaneous daily locomotion). These results suggest that *ics* mutations affect ethanol-induced behavior without generally disabling the flies.

Rsu1 Is Required in the Adult Nervous System for Normal Ethanol Sensitivity

To confirm that the reduced ethanol sensitivity of *ics* mutants was due to loss of Rsu1 protein expression, we restored expression of Rsu1 by using the Gal4/UAS system (Brand and Perrimon, 1993) and introducing a *UAS-Rsu1* cDNA transgene. We drove expression of Rsu1 in *ics*^G4 mutant flies by taking advantage of the transcriptional activator Gal4, contained within the inserted P element, which disrupts Rsu1 expression, while also expressing Gal4 under the control of the endogenous *ics* promoter and enhancers. Homozygous *ics*^G4 flies carrying the Gal4-transactivated *UAS-Rsu1* transgene showed wild-type ethanol sensitivity, and restoration of wild-type Rsu1 protein expression levels (Fig. 5.1*A*). *ics*^G4 drove expression of a *UAS-GFP* reporter in the brain, including in the mushroom bodies and neurosecretory cells of the pars intercerebralis; there were no obvious differences between *ics*^G4 mutant and wild-type flies (Fig. 5.1*B*). To investigate if *ics*^G4-driven expression in the nervous system was necessary for normal ethanol responses, we suppressed the expression of the *UAS-Rsu1* cDNA in neurons using a pan-neuronal inhibitor of Gal4, *elav-Gal80* (Yang et al., 2009). Neuronal suppression of Rsu1 expression prevented rescue of the *ics*^G4 phenotype by the *UAS-Rsu1* transgene (Fig. 5.2*A*). To ask whether exclusive expression of Rsu1 in the nervous system was sufficient to rescue the *ics* mutant phenotype, we used the neuron-specific driver *elav-Gal4* to drive expression of *UAS-Rsu1* in the *ics* mutant background. As shown in Fig. 5.2*B*, reduced ethanol sensitivity of *ics*^x5 was

restored to wild-type levels when we expressed Rsu1 exclusively in neurons. Taken together, these data indicate that Rsu1 functions in the nervous system to regulate ethanol-induced behavior.

Neurons expressing Gal4 in ics^{G4} mutant brains appeared no different from behaviorally normal $ics^{G4}/+$ heterozygotes (Fig. 5.1*B*), suggesting that Rsu1 is not needed to properly set up ethanol-response neuronal circuits. Given that *ics* mutant flies show a developmental wing blister phenotype (Kadrmas et al., 2004), it was possible that we could have missed subtle developmental defects. We therefore wished to directly test the requirement for Rsu1 in adult flies, utilizing Gal80^{ts}, which allows for temperature-dependent suppression of Gal4-driver activity (McGuire et al., 2003). Using this system, the expression of Gal4 is suppressed at 18°C, but not at 29°C. We first asked whether expression of *UAS-Rsu1* cDNA during development only was able to restore normal ethanol-induced sedation to *ics* mutant adults. We reared flies (ics^{G4} *UAS-Rsu1; Tub-Gal80^{ts}*) at 29ºC, allowing developmental expression of wild-type Rsu1, and then suppressed expression during adulthood by shifting the flies to 18°C for 3 days after eclosion. Expression of wild-type Rsu1 in this manner was unable to rescue the reduced ethanol sensitivity of ics^{G4} mutants (Fig. 5.2*C*). Conversely, when we raised flies at 18°C, blocking Rsu1 expression during development, but allowed Rsu1 expression in adulthood by shifting the flies to 29°C for 3 days after eclosion, the phenotype of ics^{G4} mutants was completely rescued to wild-type levels (Fig. 5.2*D*). These data suggest that Rsu1 functions in the adult fly to regulate normal ethanol-induced

behaviors and that Rsu1 is not required for the developmental wiring of neural circuits involved in regulating ethanol responses.

Rsu1 Functions Downstream of Integrin Signaling

Developmental experiments show that Rsu1 acts in concert with the scaffolding protein PINCH to inhibit the c-Jun Kinase (JNK) signaling pathway downstream of the integrin signaling receptor (Kadrmas et al., 2004). We therefore investigated whether perturbation of the integrin signaling pathway in *Drosophila* would alter ethanol sensitivity. As previously reported (Bhandari et al., 2009), flies heterozygous for mutations in the β-integrin encoding gene *myospheroid* (mys^{ts2}) showed increased sensitivity to the sedating effects of ethanol when compared to wild type (Fig. 5.3*A*; mys^{ts2} homozygotes are not viable). When we introduced the ics^{G4} mutation into this genetic background, the mys^{ts2} ics^{G4} double mutant flies showed the same reduced ethanol sensitivity as ics^{G4} mutant flies (Fig. 5.3*B*), suggesting that Rsu1 controls ethanol-induced behavior downstream of the integrin receptor. We also observed genetic interactions between *ics* alleles and mutants in the genes encoding PINCH and integrin-linked kinase (ILK,. Fig. 5.2C,D), further supporting our hypothesis that Rsu1 affects ethanol-induced behaviors by regulating the integrin signaling pathway.

Rsu1 Acts Upstream of Rac1 and Affects Actin Dynamics

Since Rsu1 acts in concert with PINCH to inhibit JNK activity during

development (Kadrmas et al., 2004), we tested for potential genetic interactions between mutations in *ics* and *basket* (encoding JNK). We were unable to find any such interaction, or a sedation phenotype in *basket* mutants, which is consistent with two previous studies, reporting the absence of an ethanol sedation phenotype in *basket* mutants (Corl et al., 2009; Kapfhamer et al., 2012). Aside from JNK, other downstream targets of integrin signaling include Rho-family GTPases. Depletion of human Rsu1 in a human breast cancer cell line elevates the levels of activated Rac (Rac.GTP; Dougherty et al., 2008), suggesting that Rsu1 reduces Rac1 activation. We therefore investigated whether Rsu1 functions via Rac1 to affect ethanol-induced responses. Expressing dominant-negative Rac1 in *ics-Gal4* expressing cells *(ics^{G4}/+; UAS-Rac1^{DN}/+)* resulted in increased sensitivity to ethanol-induced sedation (Fig. 5.3*B*). This increased sensitivity remained the same in the *ics^{G4}* homozygous mutant background, suggesting that Rac1 regulates ethanol responses downstream of Rsu1. We next determined whether Rsu1 physically interacts with Rac1 by co-transfecting *Drosophila* Schneider (S2) cells with FLAG-tagged Rsu1 and various Rho-GTPases tagged with yellow fluorescent protein (YFP). Immunoprecipitation with an anti-FLAG antibody pulled down both GTP-locked Rac1^{G12V} (constitutively active, CA) and GDP- bound Rac1^{T17N} (dominant-negative, DN; Fig. 5.4*A*). It did not, however, co-immunoprecipitate Rho1 (Fig. 5.4*B*) or Cdc42, suggesting that Rsu1 is a specific binding partner for Rac1 in the Rho-family of small GTPases. Our genetic data indicated that Rsu1 acts upstream of Rac1 to oppose latter's activity. We therefore hypothesized that in

the absence of Rsu1 there would be increased Rac1 activation. We found that knockdown of Rsu1 with RNAi in S2 cells (Fig. 5.3F) increased levels of Rac.GTP loading (Fig. 5.4*C*). In addition, both overexpression of Rac1[CA], and Rsu1 knockdown caused a decrease in the globular to filamentous (G/F) actin ratio (Fig. 5.4*D,E*). Taken together, these data indicate that Rsu1 binds to Rac1 and destabilizes actin filaments through Rac1 inhibition.

ics Mutants Show Increased Alcohol Preference in _Drosophila_

We next asked whether *ics* mutant flies exhibit an alcohol drinking phenotype. Flies were tested in an ethanol consumption preference assay, CAFÉ (for capillary feeder) (Ja et al., 2007, Devineni et al., 2009). Wild-type flies gradually acquire preference for ethanol over three days, showing that alcohol is reinforcing consummatory behavior (Fig. 5.5D), which likely involves reward pathways. Conversely, *ics*[G4] mutant flies showed significant naïve preference for ethanol on day 1, which remained unchanged over the 4-days of the assay (Fig. 5.5*A*). This enhanced preference in *ics* mutants was caused by an increase in ethanol consumption, whereas the total food consumption volume was no different from wild type (Fig. 5.5E). Introducing *UAS-Rsu1* driven by *ics*[G4] into the mutant flies restored this phenotype to wild type, i.e. gradual acquisition of preference over the first few days of the assay (Fig. 5.5*A,* Fig. 5.5E).

The mushroom bodies (MB) are a brain center in *Drosophila* involved in higher order processing, such as associative olfactory learning (Pitman et al.,

2009) and ethanol-reinforced odor preference (Kaun et al., 2011). We next asked whether Rsu1 was required in the MB for normal ethanol preference in the CAFÉ assay. Using a MB-Gal80 transgene, we inhibited MB expression of Rsu1 in *ics^{G4}; UAS-Rsu1* flies (Fig. 5.5F; (Krashes el al., 2007). Like wild type, these flies showed no naïve ethanol preference, but unlike wild type, they did not acquire ethanol preference over the 4-day span of the experiment (Fig. 5.5*B*). To confirm that loss of Rsu1 from the MB caused this lack of acquired ethanol preference, we knocked down Rsu1 expression specifically in adult MB. Using a mifepristone-inducible *MB-GeneSwitch* driver (Mao et al., 2004), we found that adult expression of both *UAS-Rsu1-RNAi*, as well as *UAS-Rac1^{CA}* overexpression, led to a complete loss of ethanol preference (Fig. 5.5*C*). Together, our data show that flies globally lacking Rsu1 display high naïve preference that does not change over time. Conversely, flies lacking Rsu1 only in the MB show neither naïve, nor acquired preference. Both are in contrast to wild-type flies, which show no naïve preference, but gradually acquire preference in the CAFÉ over a few days. Flies lacking Rsu1 in the MB only showed normal ethanol-induced sedation (Fig. 5.5*D*). This indicates that naïve responses to ethanol, such as naïve preference and sensitivity to sedation, are mediated by Rsu1 in neurons outside the MB, while within the MB, Rsu1 is essential for gradual acquisition of preference.

RSU1 **Genotypes Are Associated with Reward Anticipation and Alcohol Consumption in Human Adolescents**

We next sought to translate our Drosophila findings to humans. Alcohol drinking activates the reward system and alcohol preference and drinking behavior is associated with reward anticipation (Stacey et al., 2012; Nees et al., 2012; Andrews MM et al., 2011; Beck et al., 2009; Vilafuerte et al., 2012; Wrase et al., 2007; Yau et al., 2012). Reward anticipation can be reliably measured during the monetary incentive delay (MID) task (Knutson et al., 2000), where subjects must press a button upon seeing an object on screen. The form of the object determines whether subjects can accrue a large, a small, or no monetary win, if pressing the button in time. To test a possible association of SNPs in human RSU1 with reward anticipation, we measured brain activation with functional MRI BOLD responses during the MID task. We first conducted neuroimaging analyses in 1303 adolescents of the IMAGEN cohort, who were assessed at age 14 years. We observed extensive activation in the brain when comparing the anticipation of a large win vs. no win, including in the ventral striatum (VS), a region crucial for reward processing (Der-Avakian et al., 2012). In this region of interest (ROI), we detected an association of the minor T-allele of rs7078011 in RSU1 with increased VS activation, which remained significant after controlling for the 70 SNPs present at the RSU1 locus in the IMAGEN dataset (p_{10000}permutation = 0.046) (Fig. 5.6A). However, we did not detect association of rs7078011 with frequency of lifetime drinking at 14 years in the IMAGEN sample. Because rs7078011 is localized in the

7th intron of human RSU1 (Fig. 5.6B), we hypothesized that it may be a marker for an unidentified linked causal variant in the vicinity. Out of the 70 SNPs identified in RSU1 22 SNPs were in linkage disequilibrium with rs7078011. These SNPs covered the 8th exon of the gene (Fig. 5.6B; Fig. 5.7). To investigate if rare variants are present in the gene locus covered by these SNPs we analyzed whole genome sequencing data of the 8th exon in reference datasets (NHLBI and 1000 genomes phase III). Here we detected several rare variants (maf < 1%) with a predicted disruption of protein function. These are either mis-sense, i.e. rs144428707 (SNP), rs375646999 (SNP), rs375416941 (SNP), rs372364335 (SNP) and rs199904406 (SNP) (Table 5.1), or splicing related, i.e. rs373104238 (Indel). However, our datasets did not have sufficient power to allow stable association analyses of these potentially causative polymorphisms.

We therefore carried out a linear kernel-based association analysis (Bach et al., 2003) of the SNPs in strong linkage disequilibrium with rs7078011. Kernels combine the contribution of genetic variations thus enabling detection of genetic effects that cannot be represented by a single SNP alone (Yang et al., 2010). While kernel analyses do not indicate a directionality of an association they are particularly sensitive in reliably detecting associations with potentially causal rare variants. We found significant associations of the RSU1-kernel with both VS activation (mc = 0.020, p_{1000}permutations = 0.0480; Fig. 5.6A, Fig. 5.8A) and the frequency of lifetime drinking (mc = 0.020, p1000permutations = 0.0140) in the IMAGEN sample at 14 years (Fig. 5.6C; Fig. 5.9B). In order to investigate if RSU1

might be a risk factor for alcohol addiction we analyzed 1149 alcohol dependent patients and 1360 controls of Caucasian descent (see Table 5.2) from the Study of Addiction: Genetics and Environment (SAGE, (Bierut et al., 2010). We found significant association of the RSU1 kernel with alcohol dependence (mc = 0.018, p10000permutations = 5.40x10-3; Fig. 5.6C; Fig. 5.8C). We also measured association of the RSU1 kernel with alcohol drinking in 4604 adults aged 31 years of the population-based North Finish Birth Cohort 1966 (NFBC 1966; (Jones et al., 1998)). However, we found no significant association with quantity of alcohol consumption (Fig. 5.6C, Fig. 5.8D). In addition, we carried out an analysis of haplotype block 5 involving rs7078011. It is noteworthy that the allele frequencies of NFBC are very different from those of IMAGEN (p = 2.03x10-48, χ2 df=21 = 286.19) and SAGE (p = 1.35x10-52, χ2 df=20 = 303.80), whereas the latter two are very similar (p = 0.922, χ2 df=21 = 12.59) (see Table 5.3), indicating distinct genetic backgrounds of the samples. There was a nominally significant association of haplotype phase 4 (Hap4) of block 5 with increased frequency of drinking in IMAGEN sample at age 14 (p = 0.0343) and a significant association of the RSU1 haplotypes with alcohol dependence in the SAGE dataset (omnibus test p = 5.99x10-3 from 10000 permutation). Although the association of the RSU1 haplotypes with alcohol dependence in the SAGE dataset was driven by Hap3 (p = 2.71x10-3), there was a trend for an association of Hap4 (p = 0.0856) (Fig. 5.6C, Table 5.4). We also found a nominally significant association of Hap4 with quantity of alcohol consumption in the NFBC 1966 dataset at age 31 years (P=0.0360) (Fig.

5.6C; Table 5.4). Last, we evaluated grey matter volume of the VS and white matter connectivity of brain structures related to the reward system and associative learning, both known to contribute to the development of addiction (Torregrossa et al., 2011). There was neither an association of rs7078011 or the RSU1 kernel with VS volume (p1000permutations = 0.449) nor with fractional anisotropy measures of Diffusion Tensor Imaging in fiber tracts linking the hippocampus with the limbic system (fornix crescent: p1000permutations = 0.554; fornix body: p1000permutations = 0.711; VS: p1000permutations = 0.176; Fig. 5.9). This suggests that the RSU1 variants alter behavior without changing human neuroanatomy, consistent with our findings in Drosophila where no obvious developmental abnormality was observed in fly brains lacking Rsu1, thus underscoring the concordance of our Drosophila and human findings.

Discussion

Role of Rsu1 and Integrin Signaling in Ethanol Sensitivity

The Rho-family of small GTPases is known to regulate ethanol-induced behaviors (Rothenfluh and Cowan, 2013), but which upstream pathways signal to these GTPases in this context is largely unknown. In this report, we characterize the effects of *icarus/RSU1* on ethanol-related behaviors. We isolated mutations in the *Drosophila ics* gene due to their reduced sensitivity to ethanol-induced sedation. *Drosophila* Rsu1, like its human homolog RSU1, is a 32-kDa protein, with a C-terminal domain that contains seven leucine-rich repeats and binds to the integrin

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effector PINCH to inhibit JNK signaling. In flies, absence of Rsu1 leads to abnormalities during wing development and dorsal closure (Kadrmas et al., 2004). Indeed, the *ics* mutants we isolated exhibit wing blisters similar to the ones caused by the loss of integrin, PINCH, and integrin-linked kinase (ILK), suggesting that Rsu1 acts in concert with these proteins in integrin-dependent cell adhesion (Kadrmas et al., 2004). Our data indicate that in the regulation of adult ethanol behaviors, Rsu1 acts downstream of integrin to antagonize integrin signaling, as suggested by the fact that loss of Rsu1 leads to reduced ethanol sensitivity, whereas loss-of-function mutations of integrin, PINCH, and ILK result in the opposite phenotype, enhanced ethanol sensitivity. Thus, Rsu1 has modulatory roles on integrin signaling that are context-dependent. During wing development, Rsu1 mediates integrin signaling to antagonize JNK (Kadrmas et al., 2004), while in the adult nervous system, Rsu1 antagonizes integrin signaling to suppress Rac1 activity.

Rsu1 Regulates Actin Dynamics

We were unable to observe any genetic interaction between Rsu1 and JNK mutants. We therefore hypothesized that Rsu1 might act via the small Rho-family GTPase Rac1 to regulate ethanol-induced behaviors, since depletion of Rsu1 enhanced Rac1 activation and cell migration (Dougherty et al., 2008). We found that Rsu1 acts upstream of Rac1 to antagonize Rac1 activity in both flies and cell culture. Rsu1 co-immunoprecipitated specifically with Rac1 (but not Rho1, or

Cdc42) from *Drosophila* S2 cells, but did not show a preference for either GTP- or GDP-bound Rac1. Since Rsu1 does not contain a potential Rac-inactivating GTPase activating domain, we hypothesize that Rsu1 prevents Rac1 from interacting with its relevant activators and/or effectors, possibly by sequestering them or by occluding binding sites within Rac1. We show that normal ethanol-induced behaviors, including sedation sensitivity and consumption preference, require proper Rsu1 and Rac1 function in the adult nervous system. This suggests that integrin signals to Rac1 via Rsu1 to regulate actin dynamics, which is known to be required for proper synaptic function (Cingolani and Goda, 2008) as well as behavioral responses to drugs of abuse (Rothenfluh and Cowan, 2013). It also establishes integrin/Rsu1 as an important functional input into the regulation of actin dynamics with behaviorally manifest consequences.

Involvement of Rsu1 in higher behaviors

Our further characterization revealed different behavioral roles for Rsu1 in anatomically distinct neuronal circuits. For normal naïve responses to ethanol, Rsu1 functions in the nervous system outside of the MB. Absence of Rsu1 from these non-MB neurons resulted in reduced sensitivity to ethanol-induced sedation, as well as in naïve preference for ethanol in a choice assay. In contrast, loss of Rsu1 in MB led to normal naïve ethanol sedation-sensitivity and consumption preference, but caused a failure to acquire ethanol preference, suggesting that activated Rac1 in the MB prevents this behavioral plasticity. Indeed, when we

overexpressed Rac1^{CA} in adult MB, the flies failed to acquire ethanol preference. Conversely, flies lacking Rsu1 throughout the brain showed high naïve ethanol preference, suggesting that activation of Rac1 outside the MB promotes naïve preference. Thus Rsu1 has opposite effects on ethanol preference, depending on the affected circuits. This is reminiscent of mouse findings where suppression of Rac1 in the nucleus accumbens promoted conditioned place preference (CPP) for cocaine (Dietz et al., 2012), while global Kalirin7 knock-out (a Rac1 activator) led to reduced cocaine CPP (Kiraly et al., 2010). Our data expand on these findings by showing that i) similar to mammals, gene function in distinct circuits can differentially affect drug preference in *Drosophila*, ii) in addition to Kalirin7-mediated activation, integrin/Rsu1-regulated suppression is an important input into Rac1 regulation, and iii) we extend the mouse Rac1 findings from effects on cocaine-mediated reinforcement (in CPP), to voluntary drug/alcohol consumption in both *Drosophila* and people.

Previous studies have shown a remarkable conservation of genetic determinants of alcohol and substance use behavior across both species (see (Schumann et al., 2011). We investigated whether *RSU1* was involved in human alcohol drinking and reward processing behaviors by performing an analysis in human reward processing and alcohol drinking behaviors, including addiction by analyzing several datasets, including the IMAGEN adolescent imaging genetics cohort (Schumann et al., 2010), the SAGE alcohol dependence dataset (Bierut et al., 2010) and the North Finnish Birth Cohort 1966 (Jones et al., 1998). As most

other large genetic datasets, these samples have been analyzed in various different projects. This raises the questions of a potentially greater false positive rate as correction for multiple testing is usually confined to the number of test within one project. While this is a real possibility, we have mitigated against this risk by i) testing a very specific hypothesis, which has been experimentally supported in the Drosophila studies presented, and ii) validating our results across different independent datasets.

Since we were interested in investigating the genetic basis of mechanisms which convey increased risk for alcohol drinking behavior we first analyzed the population based IMAGEN sample of 14 year old adolescents who did not meet criteria for alcohol use disorders. In this sample a generic reward stimulus as presented in the Monetary Incentive Delay (MID) task might be more salient and a more reliable activator of the reward system than alcohol-specific cues. Using this approach, we first carried out single SNP analyses to identify a marker for the strongest genetic signal for VS-activation during reward anticipation in the RSU1 gene. This resulted in the detection of the association of VS-activation during reward anticipation, but not of frequency of lifetime drinking with SNP rs7078011 localized in intron 7 of RSU1.

We hypothesized that rs7078011 might be a marker of potentially causative rare genetic variants. Indeed, analyzing whole genome sequencing data we detected several rare variants in a genomic locus delineated by 22 SNPs in strong linkage disequilibrium with rs7078011, which probably impair protein function of

Rsu1. While our datasets were underpowered to carry out a genetic association analysis of the rare variants detected, we were able to carry out a kernel-based association analysis with these 22 SNPs. Using the kernel method we confirmed the association of RSU1 with VS-activation during reward anticipation in the IMAGEN dataset and we also found an association of the RSU1 kernel with frequency of lifetime drinking in the same sample. The fact that the association of rs7078011 with the investigated phenotypes was less stable than the association of the kernel is in keeping with the possibility of rare variants underlying the observed associations. When rare causal variants are present, their linkage disequilibrium with non-causal SNPs with higher frequencies might vary from sample to sample. This can be due to recurrent rare mutations or a mixture of populations with different genetic backgrounds. It is thus possible that the same rare variant can be linked with different alleles in different samples. This could lead to false negative findings if only the same SNP was analyzed. Alternatively, different rare variants within the observed gene locus might associate with different phenotypes under study. Using a kernel analysis allowed us to overcome these problems.

Our kernel analyses in additional independent datasets revealed association of RSU1 with adult alcohol dependence but not adult drinking behavior in a general population sample. As early substance use in adolescents is a risk factor for adult alcohol dependence (Gil et al., 2004), these results might indicate that the effect of Rsu1 on reward processing influence a risk drinking trajectory at very early stages

of exposure to alcohol. However, one limitation of our study is that it is not possible to unambiguously rule out an association of adult alcohol drinking in the population with RSU1. The markedly different LD structure of RSU1 in the NFBC 1966 cohort might have masked an association of the kernel. The observed nominal association of the RSU1 haplotype 4 with amount of drinking might indeed indicate a weak signal in this locus.

The haplotypes included in the kernel are distributed around exon 8, which encodes one of 7 leucine rich repeats (LRRs) found in the Rsu1 protein that are crucial for its interaction with PINCH1 (Dougherty et al., 2005). In human glioma cells, an alternative splicing site has been described, which gives rise to an exon 8-deleted splice variant of RSU1 translating into a less stable protein with reduced function (Chunduru et al., 2002). It is possible that the rare variants detected might result in an impaired interaction of Rsu1 protein with PINCH and/or decreased protein stability. This might disrupt Rsu1 function in a way analogous to the knockdown of Rsu1 in Drosophila causing the alcohol preference phenotype. However, further investigations, are required to analyze the effect of these variants on Rsu1 function, and to test their association with alcohol drinking in large meta-analyses.

Together, our data show that Rsu1 regulates reward-related behaviors, such as ethanol consumption, in flies and humans. We found no structural abnormalities associated with Rsu1 variants in either flies or humans, but show that Rsu1 is required after development, in adult flies, for normal ethanol-induced behaviors.

Our data from both species are therefore highly concordant. We hypothesize that the physiological process underlying these phenotypes is synaptic plasticity. In the integrin/Rsu1/Rac1-signaling cascade both integrin (Kramar et al., 2006) as well as Rac1 (Rothenfluh and Cowan, 2013) are known to affect synaptic structure and plasticity. Our findings thus underscore the utility of model organisms. For one, they are useful in elucidating the molecular mechanisms of genes mediating addiction-like behaviors. And second, they show remarkable predictive power with unbiased forward genetic screens in generating testable hypotheses that can be translated to human phenotypes.

Although Rsu1 functions specifically to Rac1 to affect ethanol-induced behavior, other small GTPases such as Cdc42 and Rho1 also function to affect actin dynamics and ethanol sensitivity in flies (Rothenfluh et al., 2006). In the next chapter, I will explore the effects of small GTPases, their regulators (particularly RhoGAP18B isoforms), and PAK/LIMK/cofilin signaling pathway on acute ethanol responses.

Experimental Procedures and Methods

Fly Stocks and Genetics

Drosophila melanogaster were raised in a 12:12 hr L:D cycle on a standard cornmeal/molasses diet at 25°C with 70% humidity, except for temperature

sensitive experiments, which used 18 or 29°C as indicated. *w Berlin* served as the genetic background for all experiments (unless explicitly stated), which were done with 2-7 day old flies during the light phase. The Gal4-expressing *icsG4* line was obtained through a P{GawB} forward genetic screen as described (1). Excisions to obtain *icsx5* (imprecise excision) and *icsx23* (precise excision) were carried out through standard genetic crosses using the {delta2-3} jump-starter insertion and were verified by PCR and standard DNA sequencing analyses. UAS-Rsu1 transgenics were generated by PCR amplification of LD43981, introducing an N-terminal BglII site and a C-terminal XhoI site, subcloning into pUASt vector, and injection into embryos (Duke Model System Genomics). The *Rsu1 UAS*-RNAi construct targeting the fourth, and largest exon of *Rsu1* (*UAS-Rsu1* RNAi) was amplified with primers ACAACAAGATCAGCGTAATCAGTCCGGGAA and CTTATAGGTCTCCGTTTTGAGGTAGTCGATG, and cloned into pWIZ (2). This construct was injected using standard procedures. Integrin mutants (*mys^{ts}*) were obtained from M. Grotewiel (3). All other fly lines were obtained from the Bloomington Stock center.

Fly Ethanol Behaviors

Loss-of-righting (LOR) assay was performed as described previously (1). Twenty males (except in Fig. 5.4*A*) per tube were exposed to ethanol vapor. The LOR of ethanol-exposed flies was measured during ethanol exposure every 5 min by lightly tapping the tube and then counting the flies unable to right themselves. The

time to 50% LOR (ST-50) was calculated for each exposure tube by linear interpolation of the two time points around the median and then averaged over the number of tubes. The data shown in most behavior figures were collected from assays performed on a single day, to eliminate day-to-day variability. However, all experiments were repeated on multiple days, with similar results.

Ethanol preference was performed using the 2-bottle choice Capillary Feeder (CAFÉ) assay as described (4) with some modifications. Our CAFÉ apparatus consisted of a 6 well plate with 4 small holes per well drilled for insertion of truncated pipette tips and 5 μl capillaries (VWR, Radnor, PA), and 2 damp cotton balls in between wells for humidity. Capillaries were filled by capillary action, a small mineral oil overlay was added to reduce evaporation, and the capillaries were measured and replaced daily. Preference assays with 8 males per well were conducted at 25°C and 70% relative humidity, and flies chose between liquid sucrose/yeast food with, or without 15% ethanol. For the *MB-GeneSwitch* experiment, food-deprived flies were fed with 0.5 mM mifepristone for 3 hours prior to the CAFÉ assay. For measurements of ethanol concentration, flies were frozen in dry ice and homogenized in 50 mM Tris-HCl (pH 7.5), and then assayed using a kit from Genzyme Diagnostics P.E.I Inc (Charlottetown, PE, Canada).

Ethanol Absorption

Ethanol concentration in flies was measured using the ethanol reagent kit (# 22929) from Genzyme Diagnostics. Millimolar ethanol concentration in flies was

calculated in assuming the volume of a fly to be 2μl. Flies (a total of n=3 per genotype were tested, where n=1 consisted of 90 flies) were exposed to ethanol vapors (E/A: 150/0) for various times points and sedation was monitored throughout the exposures. At the end of the exposures, flies were frozen in dry ice and homogenized

Immunohistochemistry

Immunohistochemistry was performed as described (1). Green fluorescent protein (GFP) was visualized with anti-GFP (rabbit anti-GFP, 1:250); to label relevant architectural features, the presynaptic marker mouse anti-nc82 was used at 1:40 to label general neuropil/brain structure. It was developed by Erich Buchner, and obtained from the Developmental Studies Hybridoma Bank developed under the auspices of the NICHD and maintained by The University of Iowa, Department of Biological Sciences, Iowa City, IA.

Schneider Cell Culture

Stably expressing S2-Gal4 cells were transfected with 3μg of plasmids tagged to GFP or Flag for co-immunoprecipitation assay. Gateway plasmids transfected were the constitutively active form of Rac1 (pT.wV RacG12V) and Rho (pT.wV RhoG12V), dominant negative Rac1 (pT.wV RacT17N) and Rho1 (pT.wV RhoT19N), and Flag-tagged Rsu1 (pT.wF Rsu1). Rac.GTP pull down experiments were performed with GST-PBD bait protein (EMD Millipore, Billerica, MA). Rsu1

dsRNA was made using two T7 primers to prepare a Rsu1 cDNA template for in vitro transcription using the Ambion T7 MEGAscript Kit (Invitrogen, Grand Island, NY). 50μg of Rsu1 dsRNA was added to S2 cell culture for 72 hours to deplete Rsu1 levels.

G/F-actin In Vivo Assay

G/F-actin assay was performed according to the manufacturer's instructions (G/F-actin In Vivo Assay Kit, Cytoskeleton, Denver, CO). G- and F-actin bands on western blots were scanned by densitometry and the ratios of free G-actin to actin present as F-actin were calculated.

Statistics for *Drosophila* Experiment

Statistical significance was established with one-way analysis of variance (ANOVA) tests using GraphPad Prism for Mac. Since each measurement was counted based on 20 flies, its normality is automatically fulfilled based on the central limit theorem. For the post-hoc analyses, Dunnett's Test was applied to control for the multiple comparison when several groups were compared to the same control. Error bars in all experiments represent SEM. Significance was only attributed to experimental lines that were statistically different from their respective controls, defined as $p < 0.05$. In all graphs *** = $p < 0.001$, ** = $p < 0.01$, * = $p < 0.05$.

Human Cohort

Participants were tested in eight IMAGEN assessment centers (London, Nottingham, Dublin, Mannheim, Berlin, Hamburg, Paris and Dresden). The study was approved by local ethics research committees at each site. A detailed description of recruitment and assessment procedures, as well as in/exclusion criteria, has previously been published (Schumann et al., 2010). In addition, all participants passed quality control procedures for the behavioral, Functional MRI (fMRI), genotyping and gene expression data.

Monetary Incentive Delay Task and Neuroimaging Analyses

This version of the MID task has been carried out as previously described (Schumann et al., 2010). *Monetary Incentive Delay (MID) Task*: This version of the MID task consisted of 66 10-second trials. In each trial participants were presented with one of three cues (displayed for 250ms) denoting whether a target (white square) would appear on the left or right side of the screen, and whether 0, 2 or 10 points could be won in that trial. After a variable delay (4000-4500ms) of fixation on a white cross hair participants were instructed to respond with a left or right button press as soon as the target appeared. Feedback on whether and how many points were won during the trial were presented for 1450ms after the response. A tracking algorithm adjusted task difficulty (i.e. target duration varied between 100 and 300ms) so that each participant successfully responded on ~66% of the trials. For every 5 points won the participant received one food snack in the form of chocolate

candy. Only successfully hit trials were included for analysis. *Functional MRI data analysis*: Functional MRI data were analyzed with SPM8 (Statistical Parametric Mapping version 8; http://www.fil.ion.ucl.ac.uk/spm). Slice-time correction was conducted to adjust for time differences caused by multislice imaging acquisition, all volumes were aligned to the first volume, and nonlinear warping was performed to an echo planar imaging (EPI) template. Images were then smoothed with a Gaussian kernel of 5-mm full width at half-maximum. At the first level of analysis, changes in the BOLD response for each subject were assessed by linear combinations at the individual subject level for each experimental condition, and each trial (i.e. reward anticipation high gain) was convolved with the hemodynamic response function to form regressors that account for variance associated with the processing of reward anticipation. Estimated movement parameters were added to the design matrix in the form of 18 additional columns (3 translation, 3 rotation, 3 quadratic and 3 cubic translation columns, and each 3 translations had a shift of ±1 repetition time). Single- subject contrast images were normalized to Montreal Neurological Institute space. The normalized and smoothed single-subject contrast images were then taken to a second- level random effects analysis. *Whole Brain Analysis:* As this analysis is exploratory, the voxel-wise height threshold was set at $p < 0.001$ uncorrected. Statistically significant differences between genotype groups are reported as voxel-intensity t-values for clusters at $p < 0.05$ family wise error (FWE) corrected. All analyses control for handedness, gender and imaging site. The beta values from the significant clusters were averaged across all voxels

within these clusters using the MarsBaR toolbox (http://marsbar.sourceforge.net) and the data exported for graphical presentation in MS Excel.

Region of Interest (ROI) Analysis: Using the MarsBaR toolbox (http://marsbar.sourceforge.net) the ventral striatum (VS) ROI was extracted from the anticipation of high gain vs. anticipation of no gain' contrast. The extracted ROI was based on (xyz ±15 9 -9, sphere radius 9mm; (6). The beta values were averaged across all voxels within the region and these data were exported for statistical analysis in PLINK (http://pngu.mgh.harvard.edu/~purcell/plink/).

Behavioral Characterization.

The 'Quantity of Drinking' phenotype was defined using an adapted version of the 2007 ESPAD questionnaire (www.espad.org), which assesses "the quantity of alcohol consuming on a TYPICAL DAY when you are drinking". The relevant question is only answered by individuals ever drinking, and the variable is coded in a 5-point scale ranging from 1 ("1 or 2") to 5 ("10 or more"). In our analysis, we also included individuals never drinking and assigned them the value 0.

Human Genetic Analyses

For the fMRI data, 1303 baseline adolescents at age 14 years (mean = 14.4, SD = 0.4, range: 12.9-16.4) were included in the SNP analysis. For the behavioral data, we analyzed a subset of 884 follow-up individuals at age 16 years (mean = 16.9, SD = 0.5, range: 15.3-18.7). DNA purification and genotyping were performed by

the Centre National de Génotypage in Paris. DNA was extracted from whole blood samples (~10ml) preserved in BD Vacutainer EDTA tubes (Becton, Dickinson and Company, Oxford, UK) using Gentra Puregene Blood Kit (QIAGEN Inc., Valencia, CA) according to the manufacturer's instructions. Genotype information was collected at 582,982 markers using the Illumina HumanHap610 Genotyping BeadChip (Illumina, San Diego, CA). SNPs with call rates of <98%, minor allele frequency <1% or deviation from the Hardy-Weinberg equilibrium ($P \leq 1 \times 10\text{-}4$) were excluded from the analyses. Individuals with an ambiguous sex code, excessive missing genotypes (failure rate >2%), and outlying heterozygosity (heterozygosity rate of 3 SDs from the mean) were also excluded. Identity-by-state similarity was used to estimate cryptic relatedness for each pair of individuals using PLINK software. Closely related individuals with identity-by-descent (IBD > 0.1875) were eliminated from the subsequent analysis. Population stratification for the GWAS data was examined by principal component analysis (PCA) using EIGENSTRAT software. The four HapMap populations were used as reference groups in the PCA analysis and individuals with divergent ancestry (from CEU) were also excluded. DNA purification and genotyping was performed by the Centre National de Génotypage in Paris. Details are provided in the supplementary information. In total, 70 SNPs were detected in the human *RSU1* gene, and PLINK (http://pngu.mgh.harvard.edu/~purcell/plink) was implemented in the association analysis between the candidate SNP and phenotypes as well as the corresponding permutation analysis if applicable. All statistical analyses were controlled for

gender and site, and handedness was also controlled in case the fMRI data were involved. The difference between correlation coefficient r is calculated based on the Fisher r-to-z transformation. Presented p-values were all uncorrected unless otherwise specified.

Kernel-based Association Analysis

We used the kernel-generalized variance (7) to quantify the dependency between the BOLD response and Genes in the IMAGEN samples. Statistical inference was based on a permutation procedure, both a parametric approximation of the p-value and an empirically p-value were calculated, and we report the latter one in this paper. For brief, a kernel based canonical correlation analysis (CCA) is to solve a following eigen- problem between two joint Gaussian variables *Y* and *X*:

$$\begin{pmatrix} \left(K_Y + \lambda I\right)^2 & K_Y K_X \\ K_Y K_X & \left(K_X + \lambda I\right)^2 \end{pmatrix} \begin{pmatrix} \zeta_Y \\ \zeta_X \end{pmatrix} = (1+\rho) \begin{pmatrix} \left(K_Y + \lambda I\right)^2 & 0 \\ 0 & \left(K_X + \lambda I\right)^2 \end{pmatrix} \begin{pmatrix} \zeta_Y \\ \zeta_X \end{pmatrix}$$

Where *KX* and *KY* are the Gram matrix of the sample calculated using the kernel function, and λ is a small regularization parameter to avoid over-fitting. The kernel generalized variance statistics ('regularized kernel association' would be a more preferable name) is then defined as:

$$rkassoc^{(k)} = -\sum_{i=1}^{k} \log\left(1 - \rho_i^2\right)$$

Where ρ_i is the ith leading eigenvalue of the regularized Eigen problem (minus one from the eigenvalue calculated directly from the matrices). This is justified by the fact that the rest eigenvalues converge rapidly to 0, and therefore retaining these eigenvalues will not only contribute little to the association but also sacrifice the numerical stability. The thus defined kernel generalized variance approximates the mutual information between variables *Y* and *X* to the second level when the variables in question follow arbitrary distributions and 'near independency' (see (7) for a detailed proof), and therefore $rkassoc$ *(k)* ≈ *0* if *Y* and *X* are independent. To get the p-value under the NULL hypothesis, we used the permutation procedure. The columns of *X* were permutated for *B* times, and the permuted kernel generalized variance statistics were calculated and recorded. The empirical p-value was then calculated as the percent of permuted statistics exceeding the original one. We found that a gamma fit of the permuted statistics approximates the NULL distribution of statistics quite well and is especially useful when one is performing screening where statistical correction are required. We truncated the left most 2% tail of the permuted NULL to get a stable fit of the gamma distribution. A diagnostic QQ-plot was also produced along with the approximated p-value in case of deviation from the gamma fit, which happens if inappropriate regularization parameter or kernel function is chosen, or the distribution from the original space is

too irregular given a small sample size.

Intensive numerical simulations reveal no sign of inflated false positive rate of the kernel association measure. Nevertheless, in this paper we will report both the approximate/theoretical and the empirical p-values. To control for the covariates, we eliminate the covariate effect from the original space of the data. As we expect no interactions between the covariates in this study, we enforce only the linear covariate removal procedure. More general nonlinear covariate removal scheme should be enforced when nonlinear interaction between the covariates is expected and will be detailed in a separated article for more general applications. Stimulation studies indicate that removing covariates from one side is more conservative than removing covariates from two sides, while both of them show no sign of inflated false positives.

For our current application, Gaussian kernels were used, and the determination of kernel bandwidth followed the recipe of (7), the regularization parameter was set to 0.1. We permuted the sample for 1, 000 times to get the empirical p-value. After the covariate removal procedure, the data was transformed to its rank divided by sample size before testing for the kernel association to smooth out possible outliers that might destabilize the algorithm.

Bootstrapping Process for Evaluating the Contribution from Multiple SNPs

100 bootstrapping processes were conducted with the IMAGEN data to investigate the performance of including extra SNPs in the kernel based association analysis. For each process, SNPs were permuted to break their links to the ventral striatum

(VS) BOLD response, except for SNP rs7078011. The permuted SNP set was then tested for its kernel-based association with VS. The same calculation was also performed for the non-permuted SNP set. A paired t-test was then applied on the hence generated 100paired statistical scores. A significantly higher score from the non-permuted SNP sets (one-tailed test) indicates that the SNPs other than the main SNP rs7078011 do provide extra information to the activation of VS.

North Finnish Birth Cohort 1966

A sample of 4772 individuals from the Northern Finland Birth Cohort 1966 (NFBC 1966) with genotypic and phenotypic data available was drawn from the population-based NFBC 1966 (see http://www.oulu.fi/nfbc/). Pregnant females with delivery dates in 1966 were recruited from the northern Finish provinces of Oulu and Lapland. Offspring data used here was obtained in 1997, when the cohort was 31 years of age. Frequencies of food and alcohol consumption at 31 y were ascertained as part of the larger postal questionnaire, which the study subjects returned at the clinical examination. Alcohol use questions (AUQ) were designed to measure the average frequency of consumption of beer, wine and spirits during the last year, and the usual amount of each consumed on one occasion. The amount of alcohol consumed per day was calculated using the following estimates of alcohol content (vol%): beer 4.8; light wines 5.0; wines 14.5; spirits 37.0. The subjects were then assigned to four groups by sex-specific quartiles of alcohol intake, those in the highest quartile being regarded as heavy drinkers.

A

genotype

	UAS-Rsu1	
$ics^{G4/G4}$	–	M
$ics^{G4/G4}$	√	R
$ics^{G4}/+$	–	C
$ics^{G4}/+$	√	

ST50 (min)

C M R — actin — 37kd — Rsu1

B

ant. PI

post. MB

ics^{G4}/+;
UAS-mCD8-GFP

ics^{G4}/Df;
UAS-mCD8-GFP

C

genotype

ics^{G4}/ics^{G4}
$ics^{G4}/+$
ics^{BG}/ics^{BG}
$ics^{BG}/+$
$+/+$

ST50 (min)

D

genotype

ics^{G4}/ics^{G4}
ics^{x5}/ics^{x5}
ics^{x23}/ics^{x23}
$+/+$

ST50 (min)

E

ics +   G4   x5   x23

40kD — actin
25kD — Rsu1

F

x5 del.

G4 ▼ BG ▼

ics

500bp

kurz ◄

G

ethanol (mM)

minutes of ethanol exposure

ctl
ics^{G4}

109
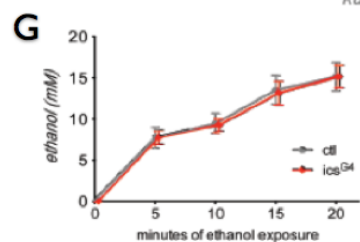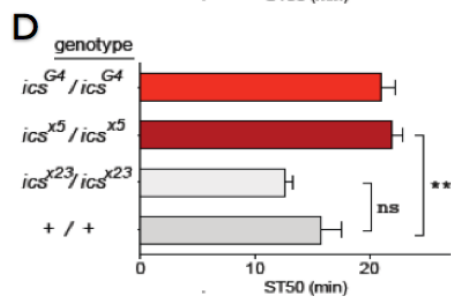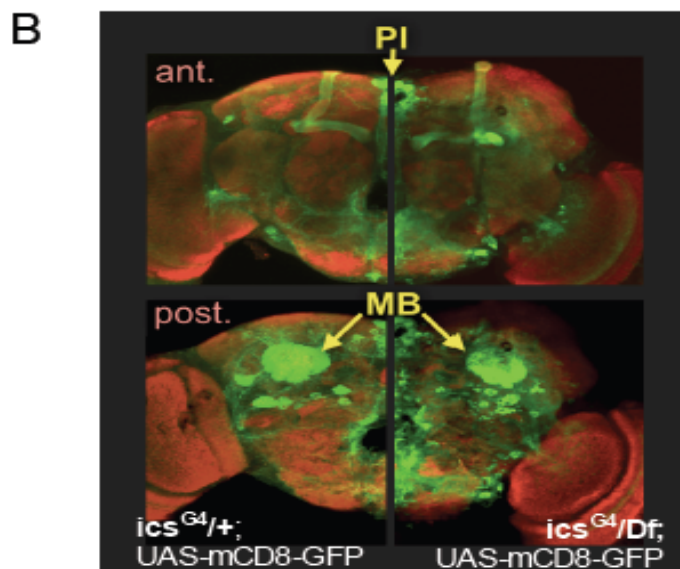
Figure 5.1. *icarus*, encoding Rsu1, is required for normal ethanol responses. In this, and the two following graphs, flies were exposed to 130/20 ethanol/air flow rate, and bars represent means ±SEM. ST-50 stands for the median sedation time; increased ST-50 indicates reduced ethanol sensitivity. (A) Mutant *icsG4* flies show reduced sensitivity to ethanol-induced sedation. This phenotype, and the loss of Rsu1 protein (inset) are rescued with expression of Rsu1 cDNA (*UAS-Rsu1*; transgene presence indicated by √ checkmark; ***p < 0.001, n = 8; C stands for control, M for mutant, and R for rescue). (*B*) Brain expression pattern of *icsG4* revealed by a membrane-bound green fluorescent protein reporter (*UAS-mCD8-GFP,* green). The picture shows an anterior (top) and posterior (bottom) confocal stack of *ics^{G4}* heterozygous wild-type (left), and homozygous mutant flies (right). Expression includes neurosecretory cells in the pars intercerebralis (PI), as well as the mushroom bodies (MB). Neuropil is counterstained with anti-Brp nc82 antibody (red). (*C*) *ics* homozygous mutant flies (*ics^{BG}* and *ics^{G4}*) are resistant to ethanol-induced sedation when compared to wild type (***p < 0.001, n = 10-17, one-way ANOVA with Dunnett's multiple comparison post-hoc test). (*D*) Precise excision of the *ics^{G4}* P-element, *ics^{x23}*, reverts the sedation resistance back to wild-type levels, while an imprecise excision, resulting in a 1.4 kbp deletion, *icsx5*, retains the ethanol-resistance phenotype (***p < 0.001, n = 8-9). (*E*) Western blot analysis showing that Rsu1 protein expression is absent in *ics* mutants *(ics^{G4}* and *ics^{x5}*), but present in the precise excision (*ics^{x23}*) when compared to controls (*ics/+*). A representative blot of 3 repeats is shown. (*F*) Schematic representation of the *ics* locus, with exons as boxes, and the open reading frame in red. P-element insertion sites are represented by triangles, and the imprecise excision *icsx5* is depicted by the interruption in the line atop (*x5 del.*). (*G*) *ics* mutants have normal ethanol absorption and metabolism. Flies were exposed to 150:0 ethanol/air, flash frozen, and their internal ethanol concentration was measured. Two- way ANOVA indicates significant ethanol increase over exposure time (*p* < 0.001, n = 4 per genotype), but no effect of genotype (*p* > 0.71).

A

elav-Gal80   UAS-Rsu1

-    + /Df   -
-    ics^{G4}/Df   -
-    ics^{G4}/Df   √
√   ics^{G4}/Df   √

0   10   20   30

ns

B   elav-Gal4

√   +   -
√   ics^{x5}   -
√   ics^{x5}   √

0   10   20   30

ST-50 (min)

***

C   Tub-Gal80^{ts} &

ics^{G4/G4}   -
ics^{G4/G4}   √
ics^{G4}/+   -
ics^{G4}/+   √

ns

test
eclosion

D   ics^{G4/G4}   -
ics^{G4/G4}   √
ics^{G4}/+   -
ics^{G4}/+   √

UAS-Rsu1

0   10   20

ST50 (min)

***

test
eclosion

111
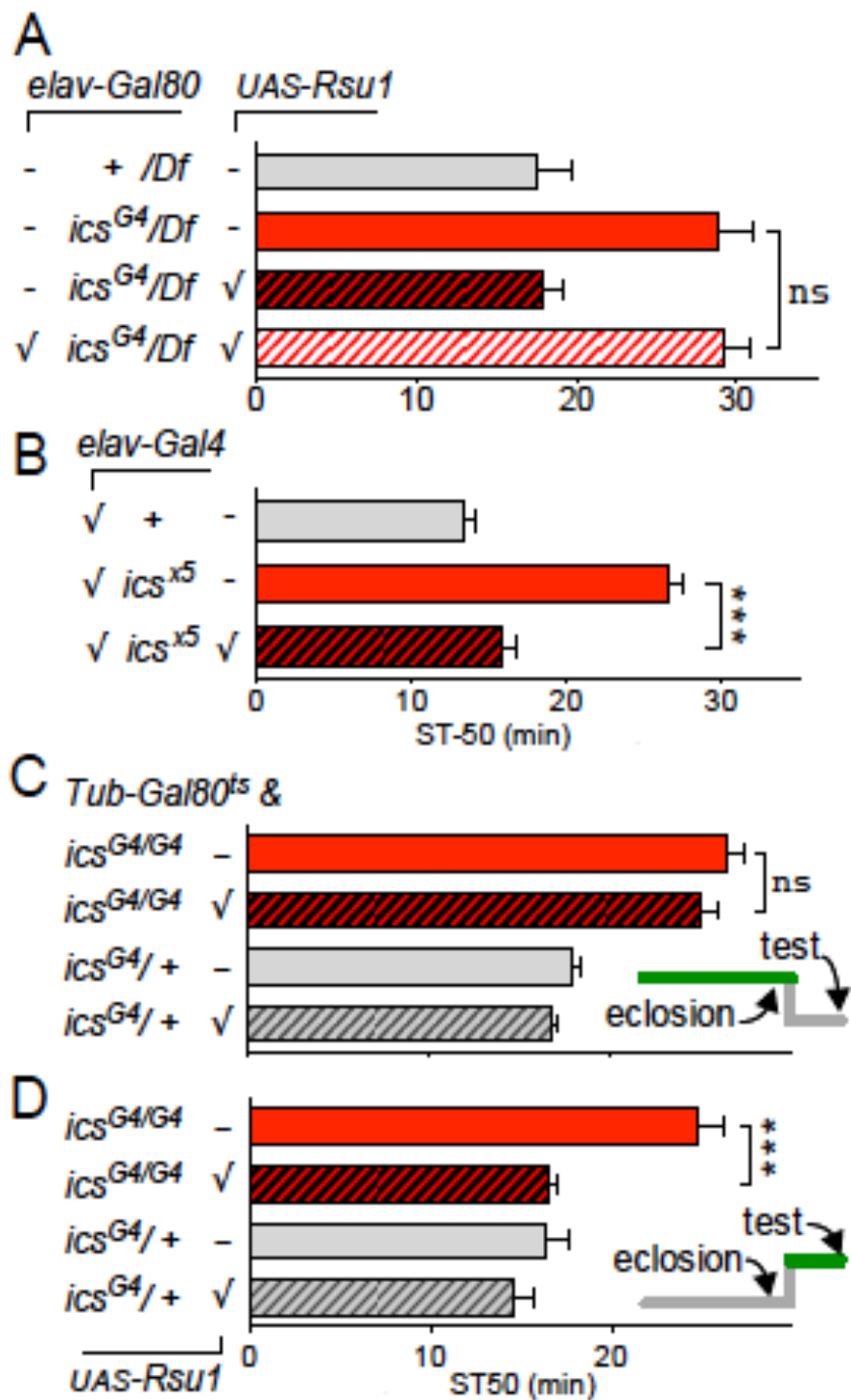
Figure 5.2. Rsu1 is required in the adult nervous system for normal ethanol responses. (*A*) Suppression of Gal4 and *UAS-Rsu1* expression in the nervous system with *elav-Gal80* abrogates the behavioral rescue (ns = not significant, *p* > 0.91, n = 6-7; *Df* represents the genetic deficiency *Df(2L)BSC147* completely removing the *ics* gene locus). (*B*) Rsu1 expression exclusively in the nervous system, via *elav^{c155}-Gal4,* completely rescues the reduced ethanol-sensitivity phenotype of *icsx5* mutant flies (***p < 0.001, n = 7-9). (*C, D*) Adult expression post-eclosion (*D*), but not throughout development (*C*), rescues the reduced ethanol-sensitivity phenotype of *ics^{G4}* mutant flies. *UAS-Rsu1* expression was suppressed using ubiquitously expressed Gal80^{ts}, which inhibits Gal4 (and therefore Rsu1 expression) at 18°C (grey, inset) but not 29°C (green, inset). Flies were kept for 3 days at the test temperature prior to ethanol exposure (ns *p* > 0.29, ***p < 0.001, n = 6-9).

A

+/+ ; +/+

+/+ ; ics^{G4}/ics^{G4}

$mys^{ts2}/+$; ics^{G4}/ics^{G4}

$mys^{ts2}/+$; +/+

ns

**

ST-50 (min)

B

+/+ ; $UAS\text{-}Rac^{DN}/+$

ics^{G4}/ics^{G4}; +/+

ics^{G4}/ics^{G4}; $UAS\text{-}Rac^{DN}/+$

+/ics^{G4}; $UAS\text{-}Rac^{DN}/+$

& $Tub\text{-}Gal80^{ts}$

**

ns

ST-50 (min)

C

elav-Gal4;

!   + ; +

!   Rsu1-RNAi; +

!   Rsu1-RNAi; $Ilk^-/+$

!   + ; $Ilk^-/+$

**

ST-50 (min)

D

elav-Gal4;

!   + ; +

!   Rsu1-RNAi; +

!   Rsu1-RNAi; $stck^-/+$

!   + ; $stck^-/+$

**

ST-50 (min)

113
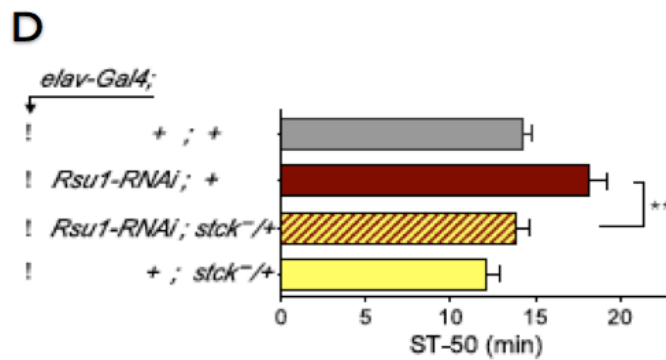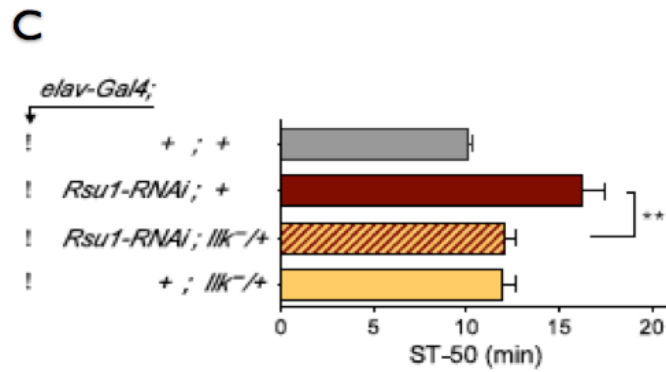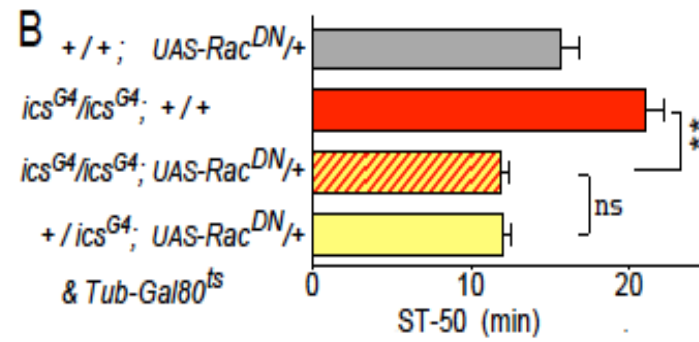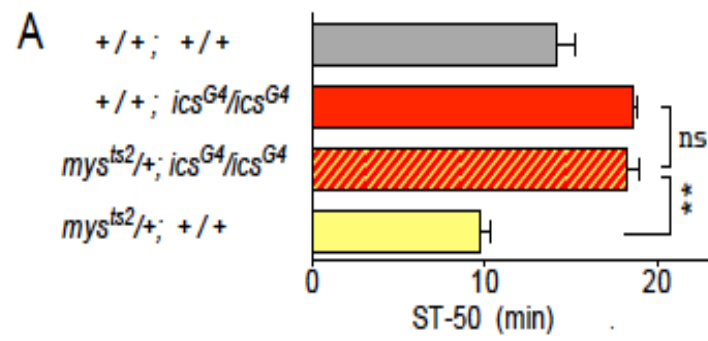
Figure 5.3. Rsu1 links ß-integrin to Rac1 signaling. (*A*) *ics*^{G4} homozygous mutants combined with heterozygous ß-integrin loss-of-function mutants (*mys*^{ts2}) are as resistant to ethanol-induced sedation as *ics*^{G4} mutants alone, indicating that Rsu1 functions downstream of ß-integrin. (**p < 0.01, ns *p* > 0.69, n = 7-9). Females were grown at 29°C for maximum *mysts2* effect. (*B*) *ics*^{G4} homozygous mutants combined with dominant-negative Rac1 (*UAS-Rac1*^{DN}) are as sensitive as *Rac1*^{DN} mutants alone, suggesting that Rac1 functions downstream of Rsu1 (***p < 0.001, ns *p* > 0.92, n = 8- 10). Unexpressed *UAS-Rac*^{DN}/+, lacking a Gal4-driver, served as a control. (C, D) Expressing *UAS-Rsu1-RNAi* in the nervous system, using elav-Gal4 as a driver leads to resistance. Introducing a heterozygous null allele of *Ilk* (encoding integrin linked kinase, (*C*), or *steamer duck* (*stck*, encoding Drosophila PINCH, (*D*) reduces the severity of Rsu1-RNAi resistance, while having no effect on their own. Alleles used were *Ilk1* and *stck3R-17*.

A

Rsu1-FLAG	-	+	+	+	-	-
Rac1^{CA}-YFP	-	-	+	-	-	+
Rac1^{DN}-YFP	-	-	-	+	+	-

blot

anti-FLAG
IP

anti-GFP (Rac1)

anti-FLAG (Rsu1)

cell lysate

anti-GFP (Rac1)

anti-FLAG (Rsu1)

B

Rsu1-FLAG	-	-	-	+	+	+
Rho1^{CA}-YFP	-	+	-	+	-	-
Rho1^{DN}-YFP	-	-	+	-	+	-

blot

anti-FLAG
IP

anti-GFP (Rho1)

anti-FLAG (Rsu1)

cell lysate

anti-GFP (Rho1)

anti-FLAG (Rsu1)

C

anti-Rac1 blot

Rac1.GTP
pull-down

total Rac1

control   Rsu1-RNAi

D

control

Rsu1-RNAi

Rac1^{CA}

genotype   G   F

anti-actin blot

E

***

G/F actin ratio

control   phalloidin   Rsu1-RNAi   Rac1[CA]

F

blot

anti-actin

anti-Rsu1

anti-GFP
(Rac1^{CA})

control   Rac1[CA]   Rsu1-RNAi

115

Figure 5.4. Rsu1 binds to Rac1 and affects actin dynamics in *Drosophila* S2 cells. (*A, B*) Rsu1 binds to both the GTP-locked forms of Rac1 (Rac1^{CA}) and GDP-locked forms of Rac1 (Rac1^{DN}, *A*), but not to Rho1 GTP- (Rho1^{CA}) or GDP-locked forms (Rho1^{DN}, *B*). (*C*) Rac1.GTP-pull down experiments shows that RNAi-mediated knockdown of Rsu1 leads to increased Rac1.GTP loading. (*D, E*) G/F-actin assay, measuring the ratio of actin in free globular to assembled filamentous form, showing that RNAi-mediated knockdown of Rsu1 causes a ~3-fold decrease in G/F actin ratio, while overexpression of constitutive active Rac1^{CA} causes a ~9-fold decrease in G/F actin ratios when compared to controls. The actin stabilizer phalloidin also decreases the G/F ratio and served as a positive control (***$p <$ 0.001, n = 4-9). (F)Cell culture expression control blots. Western blot controls showing expression of the indicated proteins from (*D, E*). All blots are representative examples of at least 3 replicates

A

B

C

D

E
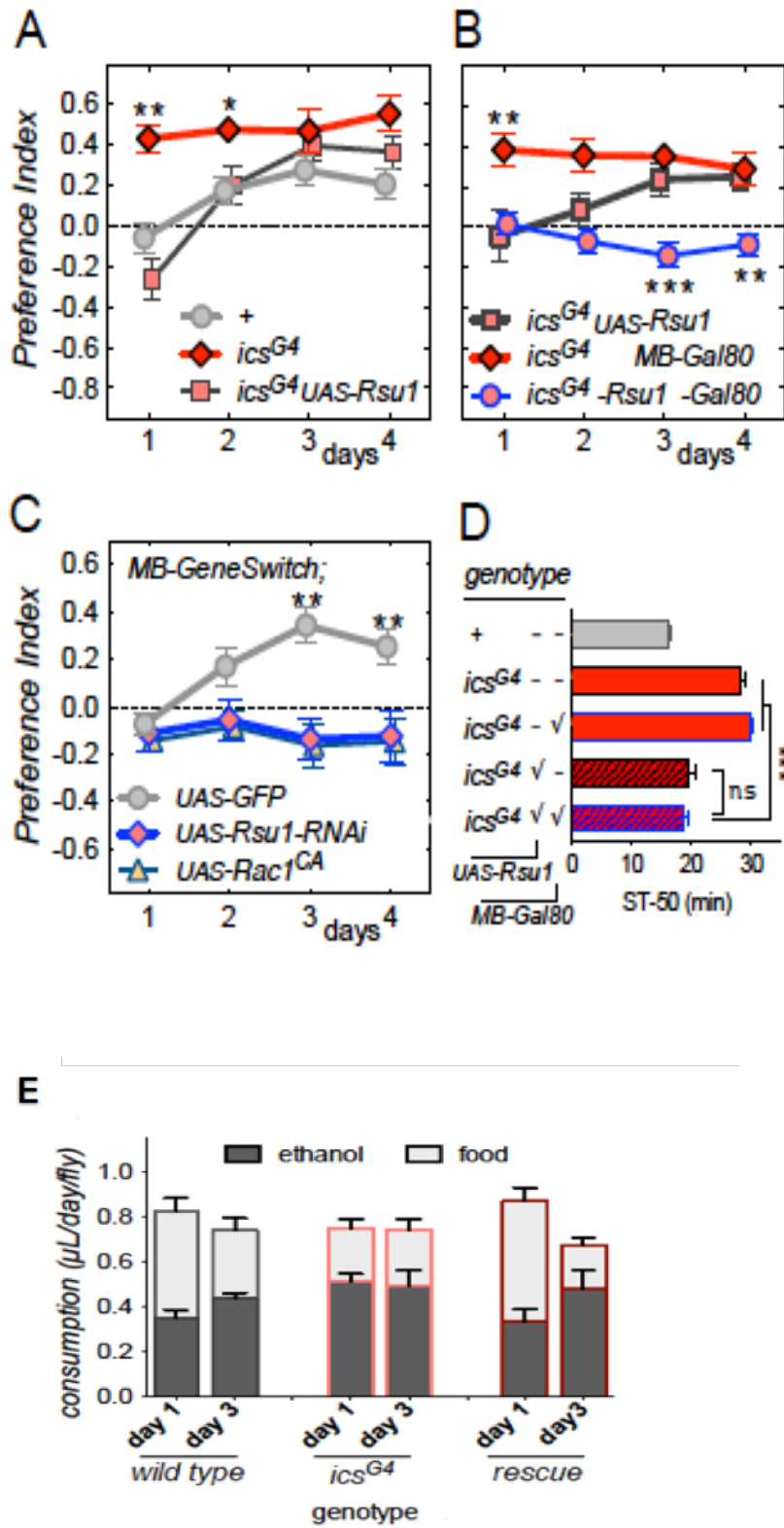
F
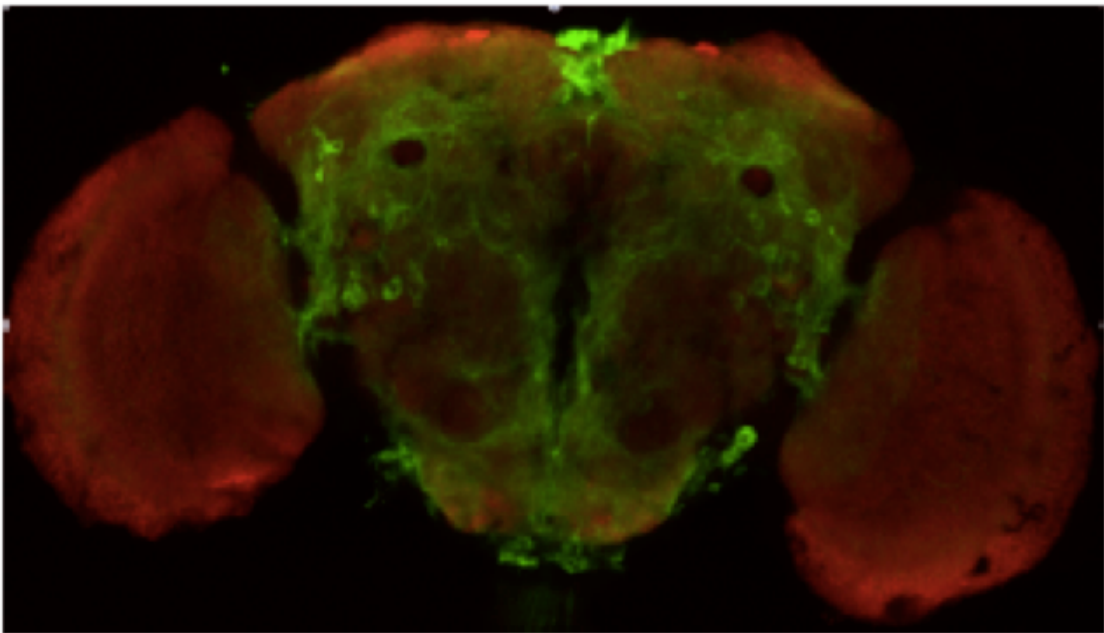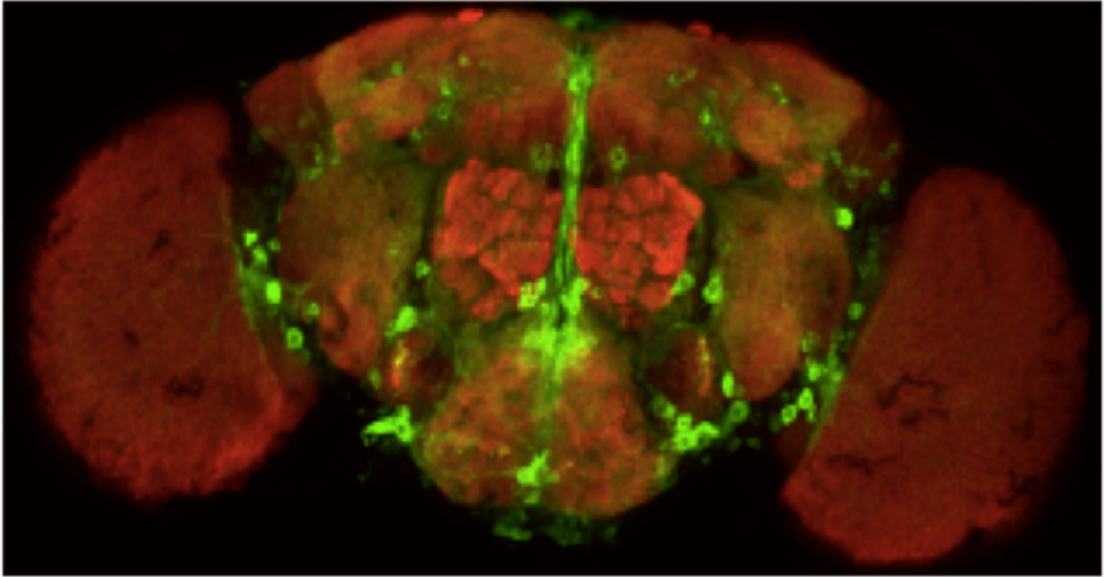
Figure 5.5. Alcohol consumption preference phenotypes in flies lacking Rsu1. (*A*) *ics* mutant flies show increased naïve ethanol preference compared to control in the 2-bottle choice CAFÉ assay. This phenotype is rescued by expression of *UAS-Rsu1* in all ics^{G4}- expressing cells (**$p < 0.01$, *$p < 0.05$ ics^{G4} vs control). (*B, D*) *ics* rescue flies lacking Rsu1 expression in the mushroom bodies (MB) only (ics^{G4} *UAS-Rsu1 MB-Gal80*) do not develop acquired ethanol preference (*B*), but have normal naïve preference on day 1 (*B*) and ethanol-induced sedation (*D*). (*C*) Adult, MB-specific knockdown of Rsu1, or overexpression of Rac1^{CA} causes loss of acquired ethanol preference. The transgenes were expressed using a mifepristone-inducible *MB-GeneSwitch* driver. (E) *ics* affects alcohol consumption. Amounts of liquid food with, and without ethanol consumed are shown. Wild-type and (ics^{G4}; *UAS-Rsu1*) rescue flies shown an increase in ethanol, and decrease in food consumption from day 1 to day 3, while ics^{G4} mutants consume high amounts of alcohol from day 1. Total amounts consumed were no different over the days or genotypes (or their interaction; two-way ANOVA, $F < 2.2$, $p > 0.14$), while ics^{G4} consumed more ethanol on day 1 than the wild-type control (one- way ANOVA with Dunnett's multiple comparison, *ics* vs. *wt*: $p < 0.01$, $q = 3.16$, $n = 20$- 29 groups of 8 flies per genotype). (F) *MB-Gal80* suppresses mushroom body expression in ics^{G4} *MB-Gal80/+; UAS- mCD8-GFP* flies. Anterior (top) and posterior (bottom) stacks are shown.

A

Anticipation Large
Win vs. No Win

- **PBR** (red)
- **VS ROI** (blue)

	rs7078011 of RSU1 (10000 Permutation)	Linear Kernel of RSU1 (1000 Permutation)
Ventral Striatum	P=0.046	P=0.048

B

RSU1 gene direction ⟶

rs7921941 rs7078011 rs12572686

NP_001072.2

RSU1

exon 8

< 20kb >
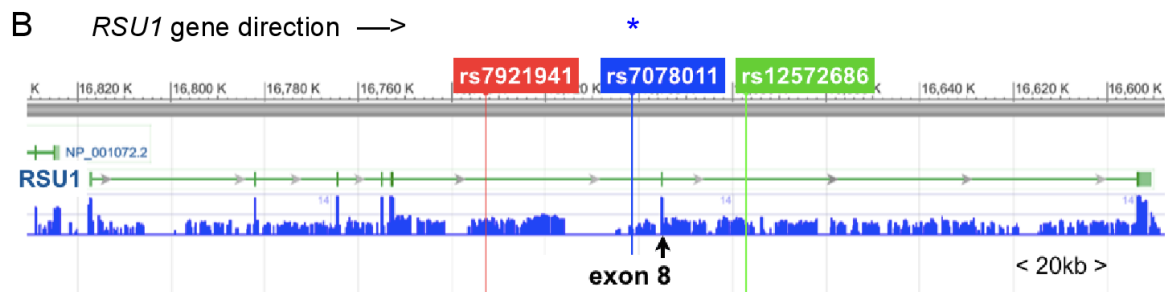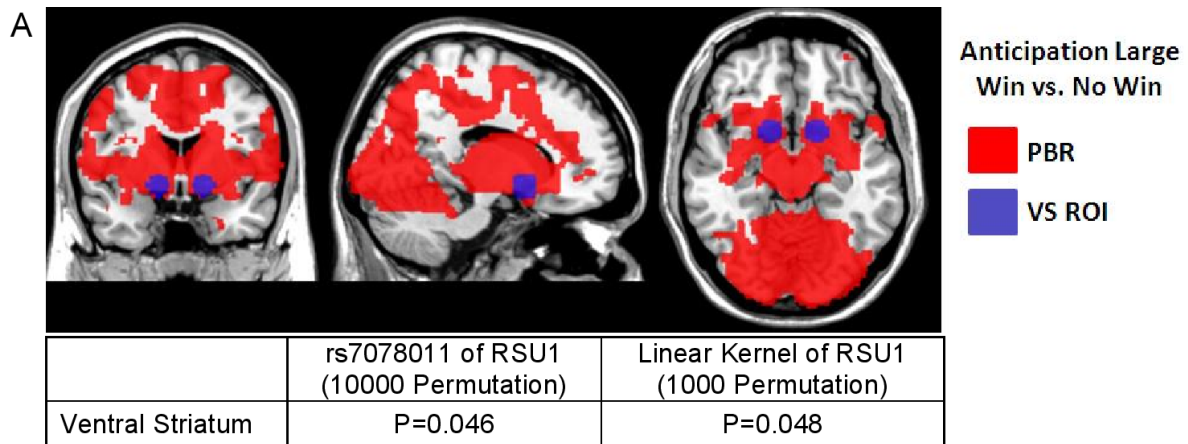
C

	IMAGEN	**NFBC**	**SAGE** (Caucasian)
	Life-time frequency of drinking (N=1908)	Quantity of alcohol consumption (g/d) (N=4604)	Alcohol dependence vs. dontrol (N=2346)
Linear Kernel Analysis	*MC=0.020 P=0.0140	Not Significant	MC=0.018 P=5.40×10^{-3}
Haplotype Analysis	Hap4 (P=0.0343)	Hap4 (P=0.0360)	Hap3 (P=2.71×10^{-3}) Omnibus (P=5.99×10^{-3})

*MC stands for generalized mean correlation as a measure of effect size.

120

Figure 5.6. Genetic studies in humans. (**A**) Whole brain analysis of reward anticipation large win vs. no win during the monetary incentive delay task shows positive BOLD response (PBR) during reward anticipation (FWE p < 0.05). The location of the VS (**±15** 9 -9; 9 mm radius) is depicted in blue. The results of association analyses between VS and RSU1 gene are summarized. (**B**) Exon/Intron schematic of **RSU1** gene. The first SNP of haplotype block 6 (rs7921941, red), the last SNP of haplotype block 5 (rs12572686, green) and the main SNP (rs7078011, asterisk, blue) is highlighted. The 8th exon is indicated with an arrow. (**C**) Summary of genetic analyses of alcohol drinking in the human datasets IMAGEN, SAGE and NFBC 1966.
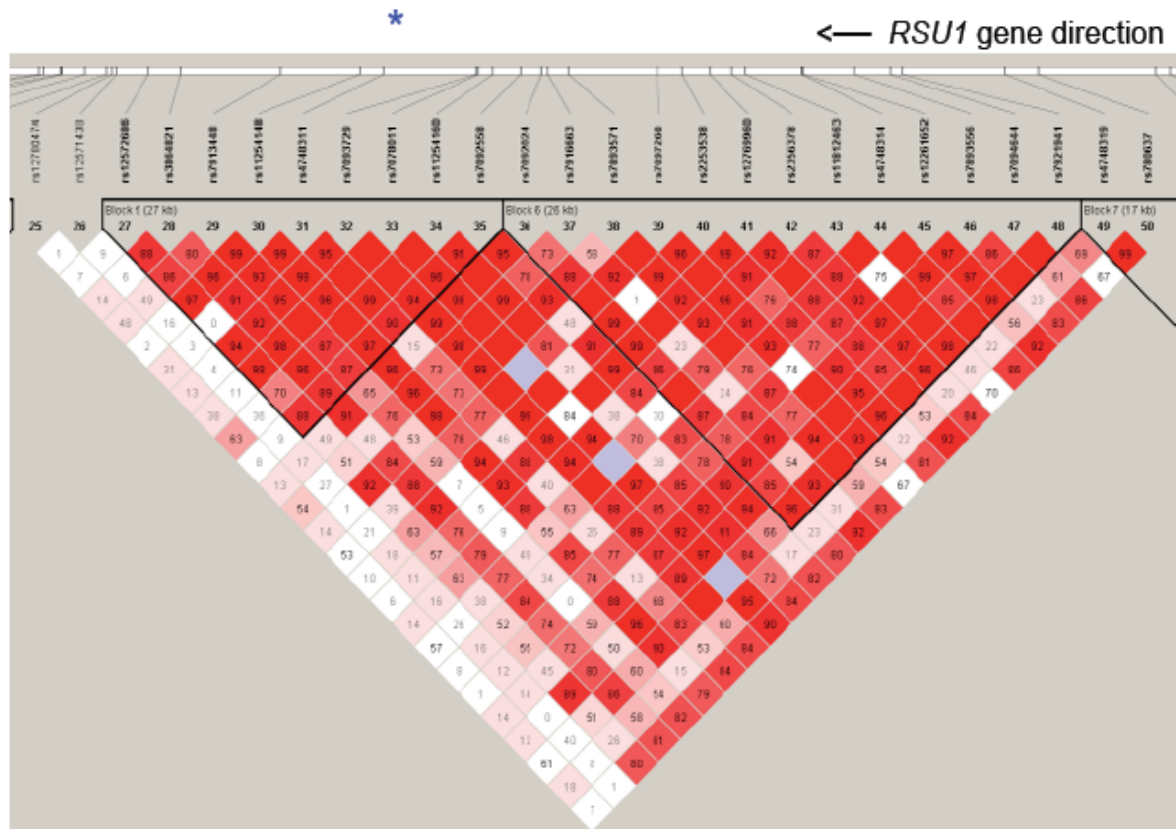
Figure 5.7. Genetic structure of haplotype blocks 5 and 6 of human *RSU1*, encompassing the 22 SNPs grouped in the kernel analysis. (*A*) Linkage disequilibrium structure of regions around SNP rs7078011 (asterisk atop) of *RSU1*, where both the adjusted linkage disequilibrium (scales as number) and the R-square (scales as color) are shown. The haplotype blocks are defined through the 'solid spine of LD' with threshold 0.80.

A

Histogram of the NULL Distribution

QQ plot of the Null Distribution

k=5, mc=0.02, p_{approx} =4.88e−02, p_{emp} = 4.80e−02

B

Histogram of the NULL Distribution

QQ plot of the Null Distribution

k=5, mc=0.020, p_{approx} =1.28e−02, p_{emp} = 1.40e−02

Histogram of the NULL Distribution

QQ plot of the Null Distribution

k=5, mc=0.018, p_{approx} =5.22e-03, p_{emp} = 5.40e-03

D

Histogram of the NULL Distribution

QQ plot of the Null Distribution

k=5, mc=0.007, p_{approx} =6.47e-01, p_{emp} = 6.28e-01

Figure 5.8. Kernel-based associations of RSU1 SNPs. (A-D) Associations in kernel-based analyses between RSU1 SNPs and ventral striatum activation in the MID task (A), lifetime alcohol consumption frequency in the IMAGEN sample at 14 years old (B), alcohol dependence in SAGE Caucasian sample (C) and alcohol consumption in the NFBC sample at 31 years old (**D**). In the histograms (top), the empirical distributions of statistics (column bars) from 1000 or 10000 permutations were plotted along with its theoretical gamma distributions (red lines), and the observed statistics were plotted as red crosses. The further those statistics are from the median, the smaller the observed p-value. The empirical and theoretical distributions were plotted against each other as the Q-Q plot (bottom), where the match between the dots (the observed quantile ratios) and the hard line (the expected quantile ratios) suggests that the observed p-values based on the theoretical distribution was reliable.

A

Histogram of the NULL

QQ plot

$mc^{(5)}=0.047129$, $p_{approx}=1.314977e-01$, $p_{emp} = 1.330000e-01$

B

Histogram of the NULL

QQ plot

$mc^{(5)}=0.042041$, $p_{approx}=5.392936e-01$, $p_{emp} = 5.270000e-01$

126

C

Histogram of the NULL

QQ plot

$mc^{(5)}=0.045273, p_{approx}=3.491091e-01, p_{emp} = 3.530000e-01$

D

Histogram of the NULL

QQ plot

$mc^{(5)}=0.041707, p_{approx}=7.767749e-01, p_{emp} = 7.550000e-01$
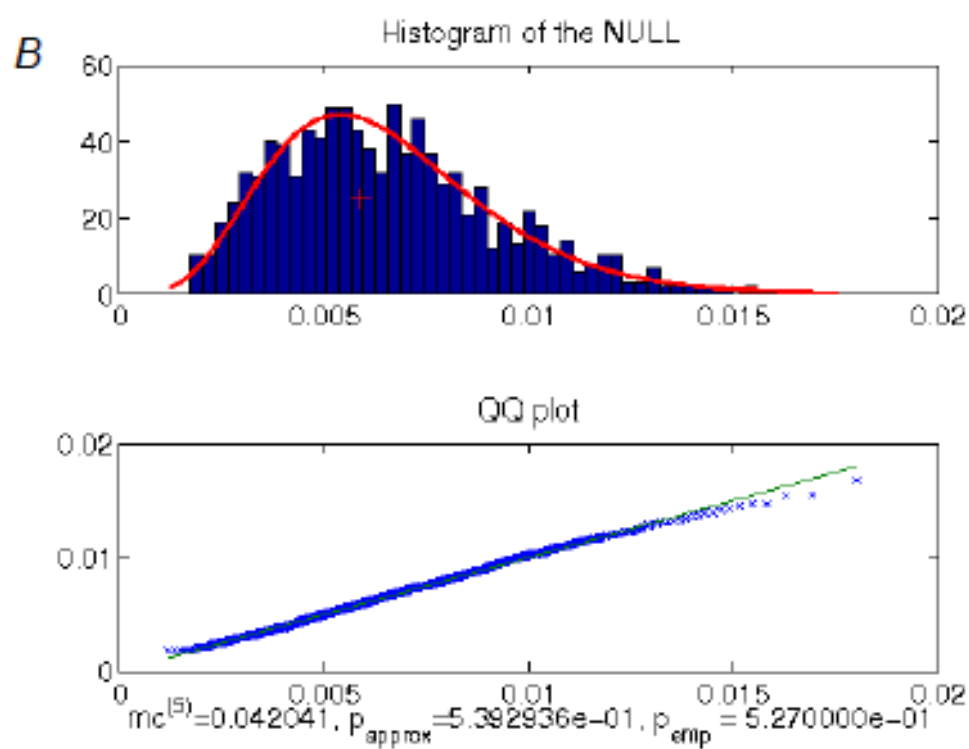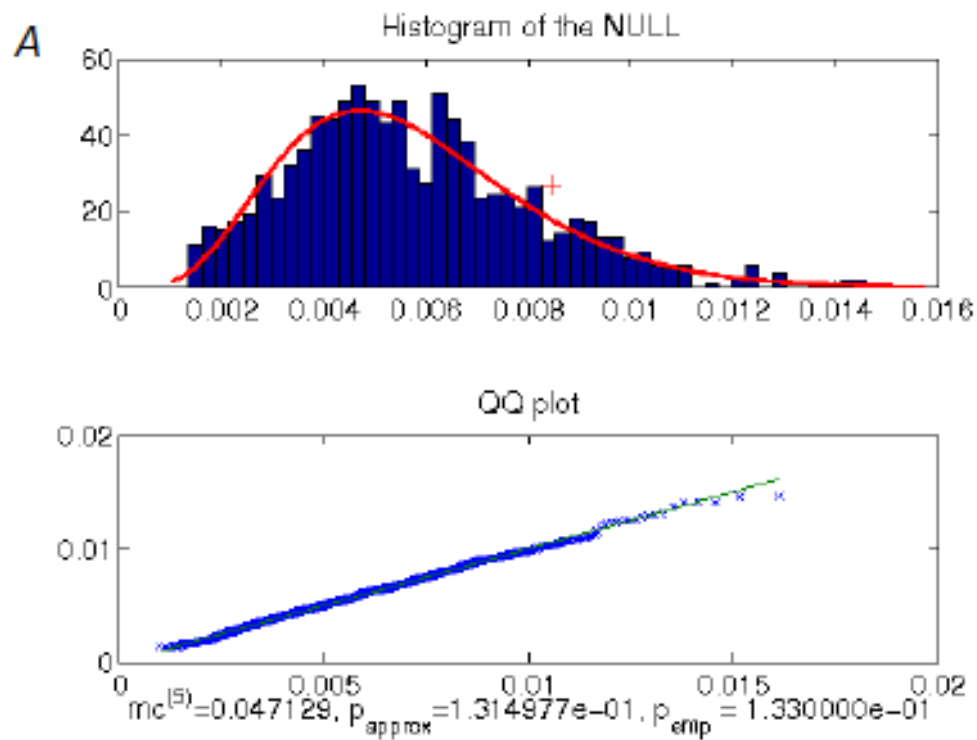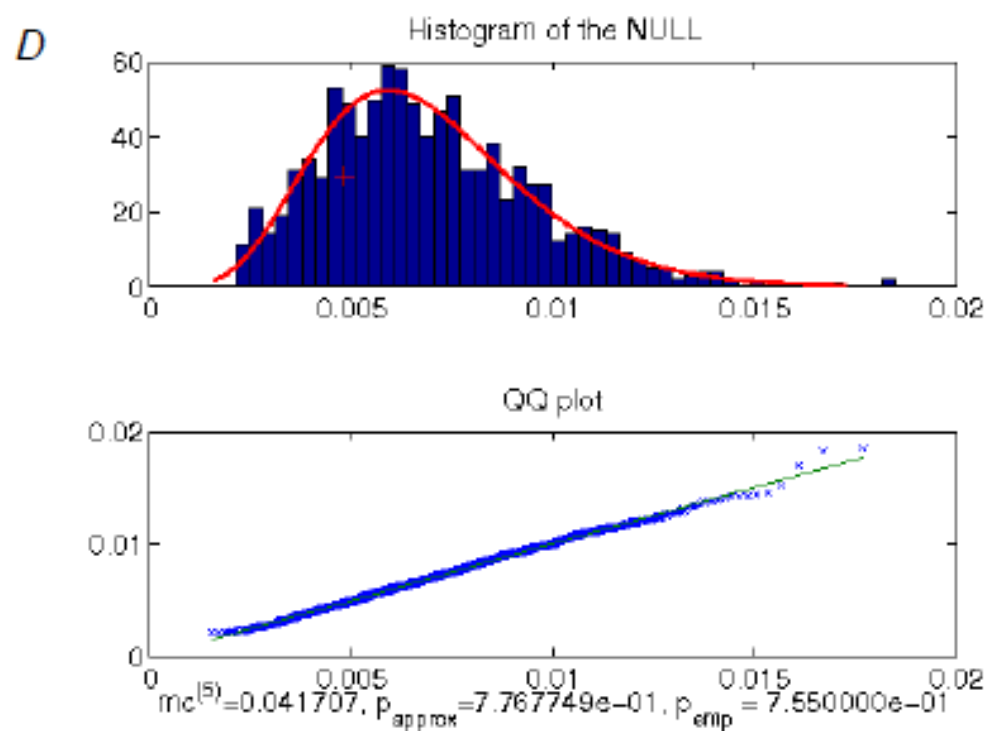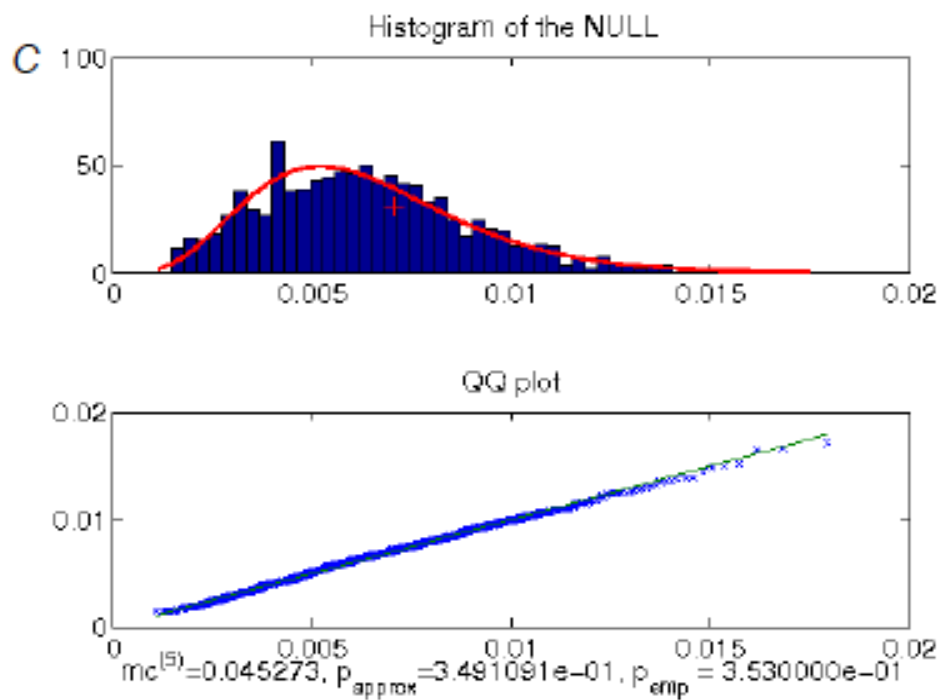
Figure 5.9. Kernel-based Associations of *RSU1* SNPs with diffusion tensor imaging data. Results for kernel based association analyses between *RSU1* SNPs and ventral striatum (VS) grey matter volume (*A*), fractional anisotropy measures of diffusion tensor imaging in fornix crescent (*B*), fornix body (*C*) and VS (*D*). In the histograms (top), the empirical distributions of statistics (column bars) from 1000 permutations were plotted along with their theoretical gamma distributions (red lines), and the observed statistics were plotted as the red crosses. The empirical and theoretical distributions were plotted against each other as the Q-Q plot (bottom), where a match between the dots (the observed quantile ratios) and the line (the expected quantile ratios) suggests that the observed p-values based on the theoretical distribution is reliable.

Miss_Sense SNP	Gene	NCBI Reference	Location_on_Protein	AA1	AA2	Probility of Disruption
rs375646999	RSU1 isoform 1	NP_036557.1	208	K	E	0.028
rs144428707	RSU1 isoform 1	NP_036557.1	230	V	L	0.001
rs372364335	RSU1 isoform 1	NP_036557.1	238	R	H	1
rs375416941	RSU1 isoform 1	NP_036557.1	242	Y	C	1
rs375646999	RSU1 isoform 2	NP_689937.2	155	K	E	0.608
rs144428707	RSU1 isoform 2	NP_689937.2	177	V	L	0.001
rs372364335	RSU1 isoform 2	NP_689937.2	185	R	H	0.999
rs375416941	RSU1 isoform 2	NP_689937.2	189	Y	C	1

Table 5.1. Predicted function of missense SNPs of 8th exon of RSU1 from PolyPhen2.

Study (origin)	N (% Women)	Age in years (SD)		% Drinkers	Geometric mean of alcohol intake among drinkers g/day/kg	
		male	female		male	female
NFBC 1966 (Finland)	5594* (51.2)	31.2(0.4)	31 (0)	90.6	0.10	0.04

*Of the 5594 participants where alcohol drinking data had been ascertained genetic data were available in n=4772 individuals.

Table 5.2. Phenotypic characteristics of the North Finnish Birth Cohort 1966.

	Frequencies			Comparison in Chi-square		
	IMAGEN (N)	SAGE (N)	NFBC (N)	IMAGEN vs SAGE	IMAGEN vs NFBC	SAGE vs NFBC
rs12572686_A	0.440 (1966)	0.427 (2544)	0.432 (4772)	0.80	0.36	0.19
rs3864821_T	0.338 (1965)	0.327 (2544)	0.278 (4772)	0.62	23.96	18.96
rs7913448_C	0.129 (1967)	0.128 (2543)	0.214 (4772)	0.01	64.77	80.52
rs11254148_C	0.386 (1966)	0.386 (2537)	0.395 (4772)	0.00	0.46	0.47
rs4748311_A	0.268 (1967)	0.276 (2543)	0.356 (4772)	0.36	48.59	47.88
rs7093729_A	0.309 (1963)	0.306 (2544)	0.263 (4772)	0.04	14.86	15.67
rs7078011_T	0.041 (1967)	0.048 (2542)	0.029 (4772)	1.32	6.02	17.05
rs11254160_A	0.093 (1967)	0.091 (2544)	0.133 (4772)	0.02	21.65	27.78
rs7092558_G	0.404 (1966)	0.395 (2543)	0.375 (4772)	0.40	4.98	2.76
rs7092024_G	0.487 (1964)	0.500 (2542)	NA	0.67	NA	NA
rs7916663_A	0.137 (1960)	NA	0.180 (4772)	NA	18.38	NA
rs7893571_G	0.332 (1963)	0.327 (2533)	0.293 (4772)	0.14	10.17	9.08
rs7097268_G	0.032 (1964)	0.031 (2544)	0.027 (4772)	0.02	1.21	1.05
rs2253538_A	0.450 (1967)	0.439 (2541)	0.468 (4772)	0.56	1.77	5.61
rs12769960_A	0.154 (1941)	0.178 (2544)	0.179 (4772)	4.38	5.71	0.01
rs2356378_A	0.471 (1967)	0.468 (2543)	0.475 (4772)	0.04	0.10	0.34
rs11812463_G	0.034 (1963)	0.032 (2544)	0.080 (4772)	0.19	46.82	64.80
rs4748314_T	0.478 (1967)	0.473 (2544)	0.489 (4772)	0.08	0.72	1.64
rs12261652_G	0.163 (1966)	0.179 (2541)	0.197 (4772)	2.05	10.57	3.38
rs7893556_T	0.460 (1966)	0.473 (2544)	0.453 (4772)	0.78	0.25	2.66
rs7094644_G	0.320 (1967)	0.316 (2541)	0.294 (4772)	0.10	4.37	3.57
rs7921941_G	0.029 (1961)	0.029 (2543)	0.026 (4772)	0.01	0.47	0.41
Sum-up Chi-square Statistics and P-values				$x^2_{df=21} = 12.59$ P=0.922	$x^2_{df=21} =$ 286.19 P=2.03x10^{-48}	$x^2_{df=20} =$ 303.80 P=1.35x10^{-52}

Table 5.3. Comparison of SNP frequencies among three datasets. For each pair of SNP frequency comparison (right), the chi-square statistic with 1 degree of freedom (df) was calculated as the square of two sample t-statistic between the SNP frequencies by definition. Under the null hypothesis, as chi-square statistics of each pair of datasets are identical and independent distributed, their sum-up will follow chi-square test with degrees of freedom equal to the number of SNPs in comparison.

	Haplotype Phase	IMAGEN		NFBC		SAGE - Caucasian	
		Freq	P	Freq	P	Freq	P
Hap1	GGTCCGCGG	0.373	0.269	0.316	0.141	0.377	0.274
Hap2	ATTACACGT	0.247	0.697	0.224	0.727	0.235	0.0903
Hap3	AGCAAGCAT	0.072	0.661	0.024	0.112	0.073	0.00271
Hap4	GGTAAGCGT	0.125	0.0343	0.018	0.0360	0.133	0.0856
Hap5	ATTACATGT	0.041	0.768	0.028	0.144	0.046	0.979

Table 5.4. Haplotype analysis of RSU1 gene with alcohol related behaviors in human. Top five most frequent haplotype block 5 phases are included in the analysis in IMAGEN sample, and then re-validate in NFBC and SAGE-Caucasian samples. The frequency of each haplotype phase and its corresponding P-value are summarized for each dataset. SNPs included in the haplotype blocks are rs12572686, rs3864821, rs7913448, rs11254148, rs4748311, rs7093729, rs7078011, rs11254160 and rs7092558 as indicated as haplotype block 5 in Fig. S6.

CHAPTER 6: RhoGAP18B isoforms act on distinct Rho-family GTPases and

regulate behavioral responses to alcohol via cofilin.

Introduction

The Rho-family of small GTPases, comprising Rac1, Rho1 and Cdc42, modulate actin dynamics in cells (Hall and Nobes, 2000). These GTPases cycle between an inactive guanosine diphosphate (GDP) form and an active guanosine triphosphate (GTP) form, which binds to and activates downstream effectors that ultimately act on the actin cytoskeleton (Malliri et al., 2002). GTPase cycling is regulated by activating guanine nucleotide exchange factors (GEFs) that facilitate the exchange of bound GDP to GTP, and GTPase activating proteins (GAPs) that stimulate hydrolysis of bound GTP to GDP, and thereby switch off the GTPases (Ehrhardt et al., 2002; Ojelade et al., 2013). Previously, we showed that loss of a specific GAP, RhoGAP18B, in *whir* mutant flies, leads to reduced sensitivity to ethanol-induced sedation in flies. Genetic experiments suggest that RhoGAP18B acts via Rac1, and/or Rho1 to modify ethanol sedation (Rothenfluh et al., 2006), but specific direct interactions between RhoGAP18B isoforms and Rho-family GTPases have not been determined.

Here, we investigated the function of the three RhoGAP18B isoforms, PA, PC, and PD in *Drosophila* cell culture. We determined effects on cell shape and actin

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polymerization, as well as binding to and regulation of Rho-family GTPases. We show specific isoform/GTPase effects, and also found RhoGAP18B-mediated regulation of the actin-severing protein cofilin. Together with our findings that adult-specific changes in cofilin modulate behavioral ethanol-sensitivity, our data indicate that RhoGAP18B shows isoform-specific regulation of subsets of Rho-family GTPases, and with it, ethanol-induced behavior.

Results

RhoGAP18B isoforms affect cell shape through their regulation of the actin cytoskeleton

Small Rho-family GTPases affect the shape and size of cell membranes by changing membrane-associated actin cytoskeleton (Meyer and Feldman, 2002; Ridley, 2006). Therefore, we investigated whether RhoGAP18B isoforms can affect F-actin mediated changes in cell shape. To do this, we overexpressed the three distinct RhoGAP18B protein isoforms (PA, PC and PD, Figure 6.1A) in *Drosophila* Schneider (S2) cells. We then characterized their effects on F-actin mediated changes in cell shape using an Alexa 568 phalloidin stain, and we did high-speed ultracentrifugation to determine globular to filamentous (G/F) actin ratios. S2 cells overexpressing either PA, PC, or PD did not show any significant changes in cell shapes and F-actin polymerization when compared to controls (Figure 6.1, D and E). However, RNAi-mediated loss of RhoGAP18B isoforms gave rise to three distinct changes in cell shape, characterized as serrate, elongate, and stellate (Figure 6.1, B and F; note that RNAi-

mediated knock down of PD also knocks down PC, since PD is fully contained within PC). Loss of PC or PD+PC predominantly led to cells having a stellate and serrate conformation, while loss of PA showed a predominantly elongated cell shape when compared to normal cells (Figure 6.1F). Additionally, RNAi-mediated loss of the common GAP domain of RhoGAP18B isoforms (GAP, Figure 6.1A) led to S2 cells having both serrate and elongated shape when compared to controls (Figure 6.1F). The loss of PC, and PC+PD also caused a significant decrease in G/F actin ratio, while PA and the common GAP did not lead to significant changes when compared to controls (Figure 6.1G). Taken together, these data show that loss of different RhoGAP18B isoforms causes distinct cellular shape and actin polymerization phenotypes.

To assess whether the different canonical members of the Rho-family of GTPases would also affect cell shape and F-actin polymerization differentially, we expressed either constitutively active (CA, GTP-locked) or dominant negative (DN, GDP-locked) forms of Rho1, Rac1 and Cdc42 in S2 cells. Expression of Rac1^{DN} and Cdc42^{DN} did not show any significant effects on cell shape and G/F actin ratios, whereas overexpression of Rho1^{DN} led to a significantly higher G/F actin ratio (Figure 6.2). Conversely, overexpression of Rho1^{CA}, Rac1^{CA} and Cdc42^{CA} showed distinct changes in cell shape, similar to loss of RhoGAP18B isoforms. S2 cells expressing either Rac1^{CA} or Rho1^{CA} were predominantly serrate and stellate (Figure 6.2B), similar to loss of the PC and PD isoforms. Overexpression of Cdc42^{CA} led to the appearance of all three cell shape changes, which included many elongated cells (Figure 6.2B), rarely seen with Rho1 or Rac1, but also found with knock down of the RhoGAP18B PA isoform

(Figure 6.1F). All constitutive active GTPases also led to a trend towards more filamentous actin, with Rac1^{CA} being the only one reaching statistical significance (Figure 6.2D). Because of the similarity of their cellular phenotypes, these data suggest that RhoGAP18B PA mainly inactivates Cdc42, while PC and PD act to suppress Rho1 and Rac1 activity.

RhoGAP18B isoforms inhibit distinct Rho-type GTPases' activity to regulate actin dynamics

As a first test of this hypothesis we determined the physical interactions of the RhoGAP18B isoforms with the different GTPases by performing co-immunoprecipitation (co-IP) assays. We co-transfected S2 cells with either FLAG-tagged PC and PD, or HA-tagged PA isoforms, together with various Rho-GTPases tagged with green fluorescent protein (GFP). HA-PA specifically pulled down Cdc42^{CA}, with little Cdc42^{DN}, and no pull down of Rho1 or Rac1 (Figure 6.3). PC pulled down all three activated GTPases (but little of the GDP-bound ones) with a preference for Rho1 and Rac1 over Cdc42, while PD only pulled down Rac1^{CA}, and to a lesser extent Rho1^{CA}. Our results are therefore consistent with our hypothesis of RhoGAP18B isoform-specific regulation of Rho-family GTPases.

We continued testing this by examining the activation and GTP-loading of the Rho GTPases as a function of losing specific RhoGAP18B isoforms. Since GAPs switch off GTPases by enhancing their GTP hydrolysis (Ehrhardt et al., 2002), we would expect increases in GTP-loading of Rho-family GTPase upon reduction of GAP proteins. Pull-

down with bait proteins specific for activated GTPases (GST-PBD from Pak1 for Cdc42 and Rac1, and GST-RBD from rhotekin from Rho1) (Malliri et al., 2002), followed by Western blotting with GTPase-specific antibodies revealed distinct GTPase activation defects. Loss of PA specifically led to activation of Cdc42 (Figure 6.4), consistent with our interaction and cell shape findings (Figures 6.1-6.3). Also consistent with the interaction data, loss of PC led to activation of all three GTPases, while loss of PD led to increased activation of Rac1 (Figure 6.4). Taken together, these results show that RhoGAP18B protein isoforms specifically regulate distinct Rho-family GTPases, with PA inactivating Cdc42, and PD inactivating Rac1. The PC isoforms exhibits less specificity, interacting with and inhibiting all three GTPases.

RhoGAP18B isoforms affect cofilin activation

One of the downstream effectors of Rho-family GTPases is cofilin, an actin binding protein that depolymerizes F-actin into its monomeric G-actin form. Inactivation of cofilin via phosphorylation can therefore lead to increased F-actin polymerization (Meyer and Feldman, 2002; Ridley, 2006; Rogers et al., 2003; Shuai and Zhong, 2010). Since RhoGAP18B isoforms function through Rho-family GTPases, we next investigated whether they affected actin dynamics by inactivating cofilin. We found that overexpressing Rac1^{CA} in S2 cells led to significant cofilin phosphorylation (P-cofilin, the inactive form), while Rho1^{CA} showed a subtle but not significant increase in P-cofilin (Figure 6.5, A and B). Next, we investigated whether RhoGAP18B isoforms function through cofilin to regulate actin dynamics by assessing if RNAi mediated knockdown of

RhoGAP18B isoforms increased P-cofilin. Western blot analysis showed that S2 cells with RNAi-mediated knock down of PC or PD had significantly more P-cofilin, similar to Rac1^{CA} (Figure 6.5, C and D). Conversely, neither loss of the PA isoform, nor Cdc42^{CA} overexpression caused a change in P-cofilin (Figure 6.5). Taken together, our data suggest that in S2 cells, RhoGAP18B-PC and PD affect cofilin activity by acting on Rac1 (and possibly Rho1) to affect the actin cytoskeleton.

RhoGAP18B functions through the LIMK/cofilin signaling pathway to affect ethanol-induced sedation *in vivo*

Loss of full length PC in the *whir^{ΔRC}* mutant of RhoGAP18B causes decreased sensitivity to ethanol-induced sedation (Rothenfluh et al., 2006). Since our data shows that the PC isoform functions through cofilin to affect actin dynamics, we sought to determine if cofilin activity is indeed relevant for ethanol-induced behaviors *in vivo*. Lin11/Isl-1/Mec3 kinase (LIMK) mediated phosphorylation and inactivation of cofilin occurs by Rho-family GTPases first activating p21-activated kinase (PAK), which in turn phosphorylates and activates LIMK. Alternatively, LIMK can be activated by Rho-associated kinase ROCK to inactivate cofilin (Ridley, 2006). To test whether LIMK was involved in ethanol-induced behavior, we first tested LIMK loss-of-function mutations (*Limk^{EY}*, (Eaton and Davis, 2005)), but found no changes in ethanol-induced sedation (Figure 6.6A). We then established *whir^1 Limk^{EY}* double mutants, to ask if a function of LIMK in ethanol sedation might be uncovered in the context of a RhoGAP18B mutant background. Loss of RhoGAP18B should lead to decreased cofilin activity, which might

be counteracted by reducing the cofilin-inactivating LIMK. Indeed, $whir^1$ $Limk^{EY}$ double mutants showed significantly less reduction of ethanol-sensitivity, when compared to $whir^1$ single mutants (Figure 6.6A), arguing that these two proteins act in opposition to regulate ethanol-sedation.

We next tested the effect of cofilin on ethanol-induced sedation. Unlike mutation of RhoGAP18B and LIMK, loss of the gene encoding cofilin (*twinstar*, *tsr*) causes lethality. We therefore tested two different cofilin loss-of-function alleles as heterozygotes and found that in the background of $whir^1$/+ flies (which show normal sensitivity to ethanol, (Rothenfluh et al., 2006), tsr^-/+ flies showed decreased sensitivity to ethanol (Figure 6.6, B and C). Since loss of RhoGAP18B-PC and PD caused cofilin inactivation in cells (Figure 6.5), this result supports our hypothesis that RhoGAP18B acts in concert with cofilin to promote ethanol sensitivity. When we assayed *tsr* mutant males that also lack RhoGAP18B ($whir^1$; tsr^-/+), they had the same reduced sensitivity phenotype as $whir^1$ mutants alone. These data show that there is a genetic interaction between *tsr* and *whir* (because the two phenotypes are not additive), and it suggests a ceiling affect, where loss of RhoGAP18-PC, with concomitant reduction in cofilin activity, can not be made any worse by additionally reducing the levels of cofilin (tsr^-/+). To confirm the involvement of cofilin in ethanol-induced sedation, we expressed a constitutively active form of cofilin (that cannot be phosphorylated/inactivated) in adult flies only, and observed the expected enhanced sensitivity to ethanol (Figure 6.6D). Adult-specific expression of a dominant-negative form of cofilin phosphatase (UAS-ssh^{DN}, expected to cause reduced activity of cofilin by increasing its phosphorylation)

led to reduced ethanol-sensitivity (Figure 6.6E). These data thus confirm a role of cofilin in ethanol-induced sedation, and together our data argue that RhoGAP18B-PC and PD modulate ethanol-induced sedation by regulating cofilin activity. The data presented here, as well as prior behavioral analysis (Rothenfluh et al., 2006), indicate that Rac1 is the major effector of cofilin activity downstream of RhoGAP18B-PC and PD, with Rho1 playing a minor role.

Discussion

RhoGAP18B isoforms act via distinct Rho-family GTPases to regulate actin dynamics

In this report, we investigated the effects of different RhoGAP18B isoforms on the actin cytoskeleton by first characterizing their effects on F-actin mediated changes in cell shape using S2 cell culture. Our data show that loss of different RhoGAP18B isoforms distinctly altered F-actin mediated changes in cell shape, which was phenocopied by different Rho-type GTPases. For instance, the PA isoform bound to Cdc42^{CA}, and loss of PA led to increased Cdc42 activation and phenocopied the effect of Cdc42^{CA} overexpression on cell shape.. Conversely, the PD isoform predominantly bound Rac1^{CA}, and to a lesser extent Rho1^{CA}, and loss of PD increased GTP loading of Rac1 and phenocopied overexpression of Rac1^{CA}, RhoGAP18B-PA thus serves as a specific GAP for Cdc42, while PD is specific for Rac1, with distinct effects on the actin cytoskeleton and cell shape The PC isoform was more promiscuous in its effects and interactions, but overall acted more similarly to PD than to PA. These findings are consistent with our *in vivo* genetic data, which suggested that loss of PC caused

reduced ethanol-sensitivity similar to overexpression of Rac1^{CA} and Rho1^{CA}, but not Cdc42^{CA} (Rothenfluh et al., 2006).

In that report we had also shown that *in vitro*, the common GAP domain preferentially acted to stimulate GTP hydrolysis of Cdc42 and Rac1, but not Rho1. This suggests that it is the distinct N-termini of PA (176 unique amino acids) and PD (460 unique amino acids) that confer GTPase-specificity in cells and *in vivo*. Interestingly, knocking down all of the RhoGAP18B isoforms in S2 cells (via RNAi targeting the common GAP domain) looked most similar to knock down of the Cdc42-specific isoform PA, while complete loss of RhoGAP18B (in the *whir*^{3} mutant) *in vivo*, resulted in the same behavioral phenotype as loss of PC, and overexpression of Rac1^{CA} and Rho1^{CA} (Rothenfluh et al., 2006). This may be a reflection of the different relative isoform expression levels, and/or mutual regulations between Rho-family GTPases in S2 cells versus neurons.

Role of LIMK and cofilin in drug-induced behaviors

Our further characterization of RhoGAP18B showed that the PC and PD isoforms function through cofilin, a downstream effector that acts to sever F-actin (Cingolani and Goda, 2008). Loss of PC and PD in S2 cells caused increased phosphorylation of cofilin, while loss of PA did not. Similarly, overexpression of Rac1^{CA}, but not Cdc42^{CA}, increased P-cofilin (with Rho1^{CA} showing a trend towards an increase). Additionally, mutations in the genes encoding for cofilin and LIMK genetically interacted with RhoGAP18B mutations, indicating that this pathway is modulating ethanol-induced

behavior *in vivo*. Indeed, adult-specific changes in cofilin activity were sufficient to alter flies' behavioral sensitivity to ethanol. This post-developmental requirement is similar to our findings with RhoGAP18B, which is also required in adults, but not throughout development, for normal sensitivity to ethanol (Rothenfluh et al., 2006). These findings argue against developmental defects, or mis-wiring causing the changes in adult ethanol-induced behavior.

Cofilin has previously been implicated in behavioral responses to cocaine in rodents. For example, cocaine conditioned place preference (CPP) was enhanced by expression of Rac1^{DN} and by constitutively active cofilin, while Rac1^{CA} suppressed cocaine CPP (Dietz et al., 2012). Viruses encoding these proteins were injected into the adult nucleus accumbens and further experiments with photo-activatable protein showed that Rac1 is acutely required during the induction of place preference (Dietz et al., 2012). Indeed, acute cocaine administration causes a transient increase in F-actin that results primarily from decreased depolymerization of F-actin via inactivation of cofilin (Toda et al., 2006). These proteins are thus acutely required during the acquisition of drug-induced memories. Our data that RhoGAP18B acts via Rac1 (and Rho1), LIMK, and cofilin to modulate ethanol-sensitivity in adult behaving flies thus expands the importance of this pathway both from rodents to flies, and also from the psychostimulant cocaine to alcohol.

Physiological role of RhoGAP18B/Rac1/cofilin signaling in behavioral ethanol responses

What are the physiological consequences that result from changes in this signaling cascade, which then alter flies' sensitivity to ethanol-induced sedation? A brief, 30 sec preexposure of cultured cerebellar granule cells to ethanol potentiates subsequent N-methyl-D-aspartate receptor (NMDAR) inhibition by ethanol. This inhibition can be prevented by the addition of phalloidin, an F-actin stabilizing agent (Popp and Dertien, 2008). Similarly, acute ethanol exposure of cerebellar granule cells leads to F-actin depolymerization, and to rundown of NMDAR currents (Offenhauser et al., 2006). Neurons lacking EPS8, a protein that regulates actin dynamics by capping the barbed end of F-actin and by activating Rac1, show a suppression of ethanol-induced decreases in both F-actin and NMDAR currents. Since EPS8 knockout mice are resistant to ethanol-induced sedation (and drink more ethanol), this suggests that stabilizing neuronal actin counteracts ethanol-induced loss of excitatory currents by stabilizing postsynaptic neurotransmitter receptors, such as NMDAR (Offenhauser et al., 2006). Indeed, recent findings in our lab suggest that ethanol causes acute sedation by silencing neuronal activity, which can be suppressed by experimental neuronal activation (Acevedo et al., 2015).

A second possibility is that this pathway is involved in synapse formation/maturation via the formation of dendritic spines. Expression of Rac1[DN] leads to a decrease in spine density, while increased Rac1 activity causes an increase in spine density in rat hippocampal neurons in culture (Impey et al., 2010; Tashiro et al.,

2000). More Rac1 activity could thus lead to more/stronger synapses, reducing the sensitivity to ethanol-induced neuronal inhibition and with it sedation. Recent findings from our lab, showing a mutual correlation of S6 kinase activity and resistance to ethanol-induced sedation, are consistent with this idea. Increased activity of S6k causes both resistance to ethanol-induced sedation (Acevedo et al., 2015), as well as increased synaptic strength (Knox et al., 2007), and synaptic size and arborization (Martin-Pena et al., 2006). The RhoGap18B/Rac1/cofilin pathway could therefore reduce flies' sensitivity to ethanol-induced sedation by strengthening the connections in the neuronal pathways that mediate ethanol sensitivity. Future experiments will explore these possibilities in more depth.

In this chapter, I strengthened the connections of small GTPases and Pak1/Limk1/cofilin signaling pathway in affecting ethanol sensitivity. But what about alcohol drinking/preference, and the neuro-circuits they are required in for drinking. To answer these questions, and to reduce the significant amounts of hands-on time for each genotype assayed, I and other members in our lab developed a high-throughput method of screening genes. I explain this modified form of the Café in the next chapter.

Experimental Methods

Cell Culture

Drosophila S2-Gal4 cells were maintained at 26°C either in Schneider media (Gibco/Life Technology, Grand Island, NY, USA) containing 10% Fetal Bovine Serum (FBS), or serum free media (SFM). Constructs were made using Gateway cloning with clonase (Invitrogen/Life Technology, Grand Island, NY, USA) and transfected using the Effectene transfection kit (Qiagen, Valencia, CA, USA). Transfections were conducted with one or more Gateway pT.UAS constructs [GFP-Rac1G12V (Rac1^{CA}), GFP-Rho1G14V (Rho1^{CA}), GFP-Cdc42G12V (Cdc42^{CA}), GFP-Rac1T17N (Rac1^{DN}), GFP-Rho1T19N (Rho1^{DN}), GFP-Cdc42T17N (Cdc42^{DN}), Flag-RhoGAP18B-PC (PC), Flag-RhoGAP18B-PD (PD), HA-RhoGAP18B-PA (PA)] depending on the experiment. Anti-PC, anti-PD and anti-PA RNAi was generated using the Megascript T7 kit (Ambion/Life Technology, Grand Island, NY, USA) from pENTR gateway cloned constructs made with isoform specific primers and cells were treated daily with 5mg dsRNAi for three days. RNAi primers PC+ (CCAAAGAGCGTACCAGCGCGCGATCC); PC- (CAACCACCGATCAACGGTTATCGGCGA); PD+ (GCTCTCCAAGCGGCGGCGG); PD- (AACCACCAGCACAACCCCACGCCG); PA+ (ATGGCCGGCGATACGGA); PA- (ATGCTGGATCTGACCTCCAACCAT); GAP+ (GATGACAAGAAGTCCATCAAG); GAP-

(GTTCCACGTTTCGTGGTC).

G/F actin Assay

G/F actin assay was performed according to the manufacturer's instructions (G/F actin In Vivo Assay Kit, Cytoskeleton, Denver, CO, USA). G- and F-actin bands on western blots were scanned by densitometry and the ratios of free G-actin to actin present as F-actin were calculated.

GTPase Activity Assay

Rac1.GTP/Cdc42.GTP levels were measured using a specific Pak1-PBD (#14-864, EMD Millipore, Billerica, MA, USA) conjugated to GST, then pull-down using GST-agarose beads and compared to total Rac1 (mouse anti-Rac1, #MAB3735, EMD Millipore, USA) or total Cdc42 (mouse anti-Cdc42, Developmental Studies Hybridoma Bank, Iowa City, IA, USA) in 3% lysate for at least three separate samples. Rho1.GTP levels were determined using pull-down with Rhotekin Agarose beads (#NC9954380, Thermo Fisher Scientific, USA) and compared to total Rho1 (mouse anti-Rho1, #p1D9, Developmental Studies Hybridoma Bank, Iowa City, IA, USA) in 3% lysate for at least three separate samples.

Co-immunoprecipitation assays

Co-IPs were conducted on the double transfected cell cultures washed with standard Phosphate buffer saline (PBS) and lysed in IP Buffer (50mM Tris-Base ph 7.4, 50mM

sodium chloride, 1% TritonX-100, 4mM magnesium chloride and protease inhibitor mixture tablets (Roche Life Science, Indianapolis, IN, USA). The supernatant was then added to FLAG-beads (Sigma-Aldrich, Inc., St. Louis, MO, USA) or HA-beads (#11815016001, Roche Life Science, Indianapolis, IN, USA) for 4 hours washed in PBS with equal volume of 2x Lämmli sample buffer added before western analysis (mouse anti-GFP, 1:1000; #MS-1315 Thermo Fisher Scientific, USA), rabbit anti-FLAG (1:1000; #F7425 Sigma-Aldrich, MO, USA), mouse anti-HA (1:5000; #H9658 Sigma-Aldrich, MO, USA). Westerns were done in triplicate to compare levels of Phospho-cofilin (#11139, Cell Signaling, Danvers, MA, USA) and cofilin (#21164, Cell Signaling, Danvers, MA, USA) from different transfected cell samples and were quantified using densitometry.

Cell Staining

RNAi treated cells were placed on poly-L-Lysine coated coverslips, fixed with 4% paraformaldehyde, blocked for 1 hour with standard PBS containing 10% normal goat serum (NGS) at room temperature (RT), then stained with Alexa Flouro 568 phalloidin (1:1000; #A12380, Molecular Probes, Inc., Oregon, USA) in PBS with 10% NGS for 45 min at room temperature (RT). A minimum of eight frames of fluorescence micrographs was taken containing on average 30-40 cells counted and categorized for cell shape for each treatment. For the transfected cells, the appropriate primary antibody: mouse anti-GFP (1:200; #MS-1315 Thermo Fisher Scientific, USA), rabbit anti-FLAG (1:200; #F7425 Sigma-Aldrich, Missouri, USA), mouse anti-HA (1:200; #H9658 Sigma-Aldrich, Missouri, USA) was added overnight in PBS with 10% NGS at 4°C. Cells were next

incubated in PBS containing 10% NGS and secondary anti-mouse or anti-rabbit FITC (1:200) antibody. After two hours, cells were washed and stained with Alexa Flouro 568 Phalloidin (1:1000; #A12380, Molecular Probes, Inc., Oregon, USA) in PBS with 10% NGS for 45 min at RT before mounting. Cells that were FITC-positive were counted and characterized.

Fly Stocks and Genetics

Drosophila melanogaster were raised in a 12:12 hr Light/Dark cycle on a standard cornmeal/molasses diet at 25°C with 70% humidity, except for temperature sensitive experiments, which used 18 or 29°C as indicated. w^{1118} served as the genetic background for all experiments (unless explicitly stated), which were done with 2-7 day old flies during the light phase. The RhoGAP18B mutant ($whir^1$) and UAS transgene constructs of all RhoGAP18B isoforms were previously described (Rothenfluh et al., 2006). All other fly strains used in this manuscript were obtained from the Bloomington Stock center.

Fly Ethanol Behaviors

Loss-of-righting (LOR) assay was performed as described previously (Rothenfluh et al., 2006). Twenty males per tube were exposed to ethanol vapor. The LOR of flies was measured every 5 min during ethanol exposure by lightly tapping the tube and then counting the flies unable to right themselves. The time to 50% LOR (ST-50) was calculated for each exposure tube by linear interpolation of the two time points around

the median and then averaged over the number of tubes. The data shown in most behavior figures were collected from assays performed on a single day, to eliminate day-to-day variability. However, all experiments were repeated on multiple days, with similar results.

Statistical Analysis

Data were analyzed using Prism, version 6.00 (Graph Pad Software, La Jolla, CA, USA) or IBM SPSS Statistics for Windows, version 21.0 (IBM Corp., Armonk, NY, USA). Analysis of Variance (ANOVAs) followed by Dunnett's post-hoc comparisons to S2-Gal4 control cells when appropriate was conducted. P values less than 0.05 (*p <0.05) were considered significant.
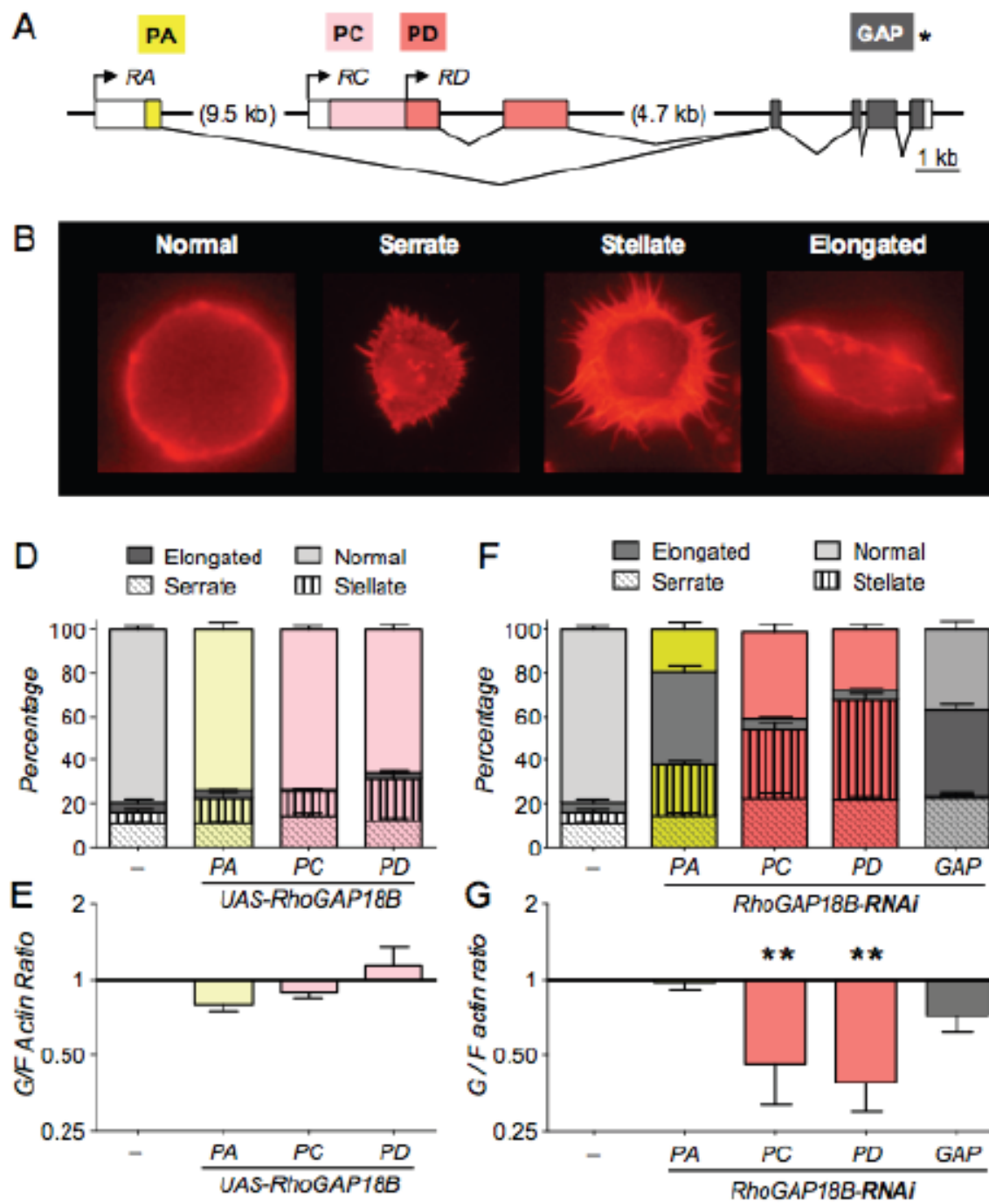
Figure 6.1. Loss of RhoGAP18B affects F-actin and cell shape structure. (A) Schematic of the *Drosophila* RhoGAP18B gene structure, with boxes representing exons. Transcripts, *R*, are indicated in italics, coding regions/proteins are indicated in color, labeled above at their N-termini. PD is fully contained within PC, and all three isoforms share a common C-terminus, which contains the GAP domain. (B) Examples of F-actin mediated changes in cell shape resulting from genetic manipulation of RhoGAP18B isoforms, classified as serrate, stellate, and elongate. Representative examples of cells after staining with Alexa Fluor 568 phalloidin. (D and F) Graph showing percentage of S2 Gal4 cells that are serrate, stellate, elongate, or normal when RhoGAP18B isoforms are overexpressed (D) or knocked down with RNAi (F). (E and G) Graph showing that overexpression of RhoGAP18B isoforms in S2 Gal4 does not lead to significant changes in G/F actin ratios when compared to controls (E), whereas RNAi-mediated knock down of PC and PD causes a significant decrease in G/F actin ratios (G). Knock down of PA, or all isoforms through targeting the enzymatic GAP domain does not cause significant G/F actin changes. One-way ANOVA with Dunnett's test compared to S2 Gal4 cells ($n = 3$-5, $**p < 0.01$).
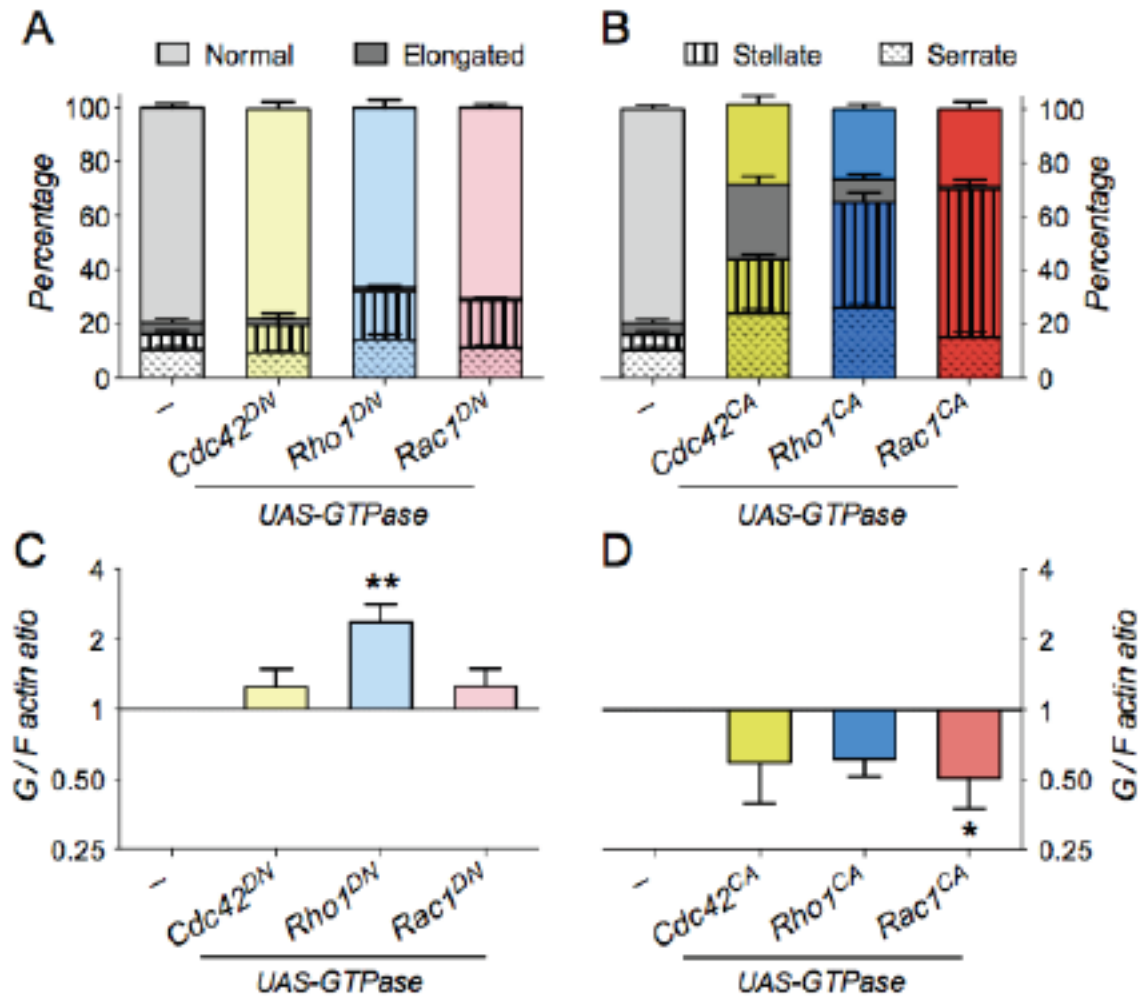
Figure 6.2. Characterization of Rho-family GTPases' effect on F-actin and cell shape. (A and B) Graph showing percentage of S2 Gal4 cells that are normal, elongate, stellate, or serrate, when dominant negative (DN, panel A), or constitutively active (CA, panel B) forms of Rho-family GTPases (Cdc42, Rho1 and Rac1) are expressed. (C and D) Graph showing changes in G/F actin ratios of S2 Gal4 cell expressing Rho-family GTPases. Expression of the dominant negative forms of Rho-family GTPases had subtle effects on cell shape (A) but did not cause significant G/F actin changes (except Rho1[DN]) when compared to controls (C). On the other hand, expression of the constitutively active forms of Rho-family GTPases caused greater effects on cell shape (B) and decreased G/F actin ratios with only Rac1[CA] showing significance. One-way ANOVA with Dunnett's post-hoc test compared to S2 Gal4 cells (n = 3-5, *$p < 0.05$; **$p < 0.01$).
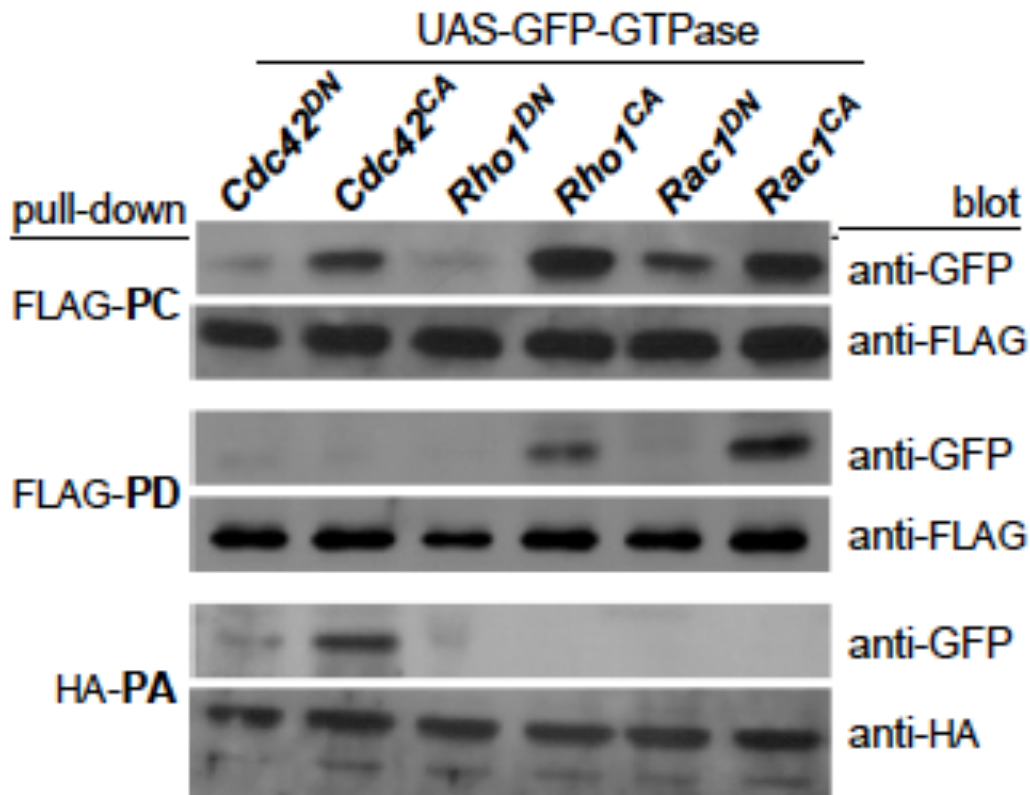
Figure 6.3. RhoGAP18B isoforms bind different members of the Rho-family of GTPases. Co-immunoprecipitation blots showing pull-down of GFP-tagged Rho-type GTPases with FLAG-tagged PC, FLAG-tagged PD, or HA-tagged PA. Rho-type GTPases pulled down with anti- FLAG or anti-HA beads was then detected with anti-GFP antibody. All isoforms preferentially bind to the constitutive active forms of Rho-family GTPases. PA binds to Cdc42 only; PD binds Rac and, to a lesser extent, Rho; and PC binds to all three GTPases. Representative blots of 3-5 independent experiments are shown.

A

RhoGap18B-**RNAi**

	ctl.	PA	PC	PD

Cdc42.GTP

total Cdc42

B

Rho1.GTP

total Rho1

C

Rac1.GTP

total Rac1

D

GTP-loading

Cdc42   Rho   Rac

fold activation

ctl.   PA   PC   PD
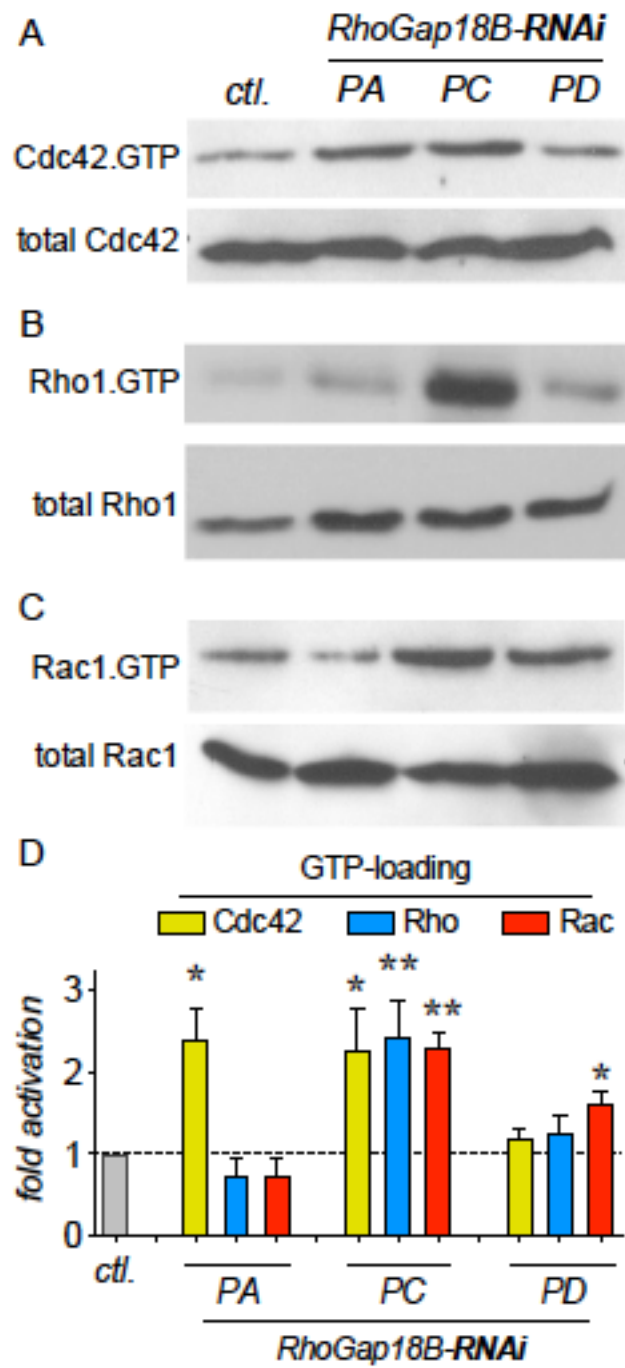
RhoGap18B-**RNAi**

Figure 6.4. RhoGAP18B isoforms inhibit different members of the Rho-family of GTPases. (A-C) Western blots of GTPase activation experiments. GTP-loaded GTPases were pulled down with Pak-PBD for Cdc42 and Rac1 and Rhotekin for Rho1 and blotted with anti-GTPase antibodies. Incubation of cells with isoform-specific RhoGAP18B RNAi is indicated at the top. Representative blots of 5 independent experiments are shown. (D) Quantitation of active/total GTPase, normalized to untreated S2 Gal4 cells, suggests specific (PA-Cdc42, and PD-Rac1), as well as general (PC) GTPase activating activities. One-way ANOVA with Dunnett's post-hoc test compared to S2 Gal4 cells ($n = 5$-7, $^*p < 0.05$; $^{**}p < 0.001$).
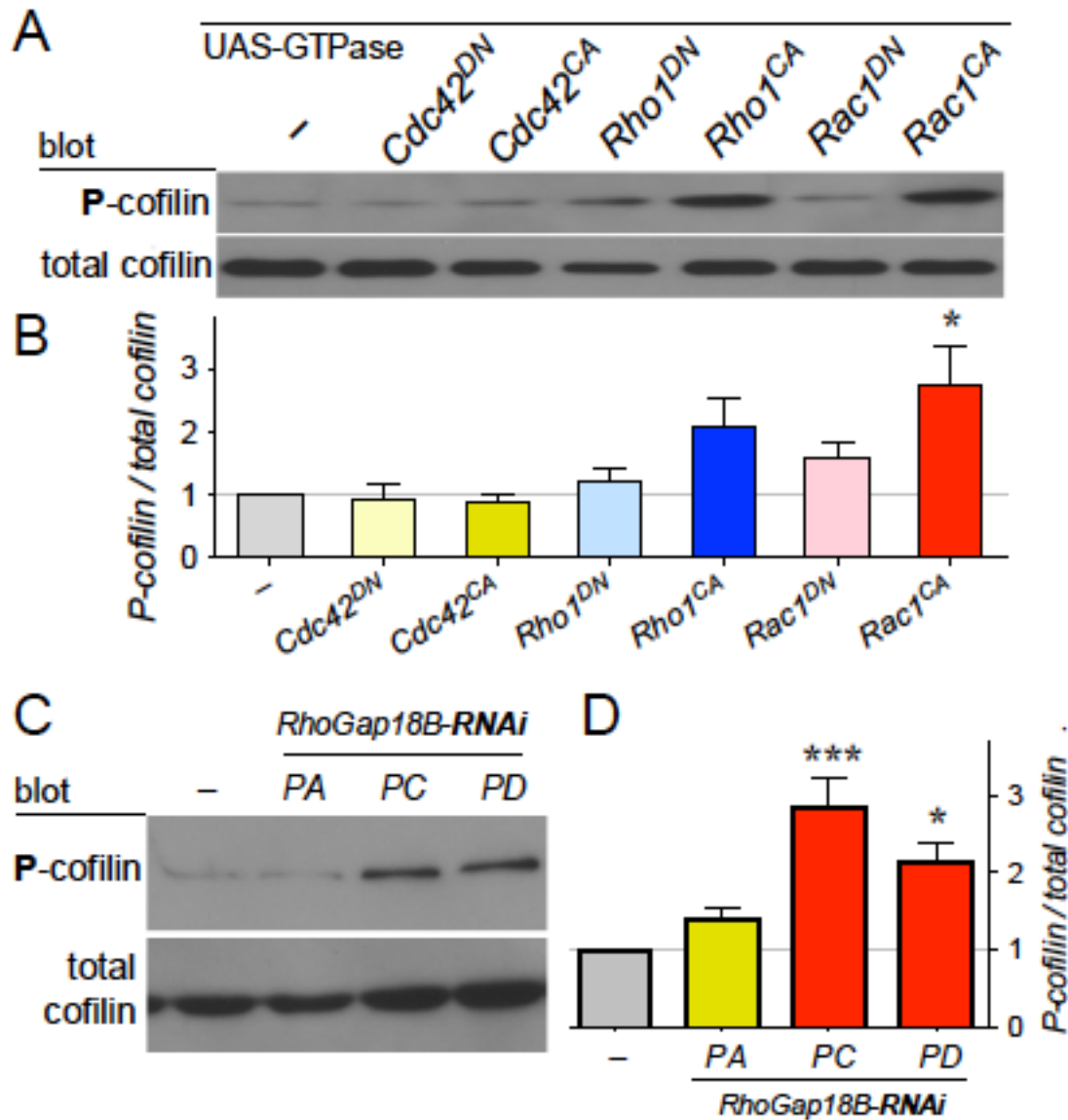
Figure 6.5. RhoGAP18B isoforms affect cofilin phosphorylation. (A) Anti-cofilin western blots from cells expressing different Rho-family GTPase constructs, indicated atop. (B). Quantitation indicates a trend towards increased P-cofilin with Rho1^{CA}, and a significant increase with Rac1^{CA}. One-way ANOVA with Dunnett's post-hoc test compared to S2 Gal4 cells (*$p < 0.05$; ***$p < 0.001$). (C) Western blot with anti-phospho-cofilin (P-cofilin), and total cofilin of cells treated with RhoGAP18-RNAi. (D) Quantitation from (C) shows that knockdown of the PC and PD isoforms leads to increased P-cofilin.
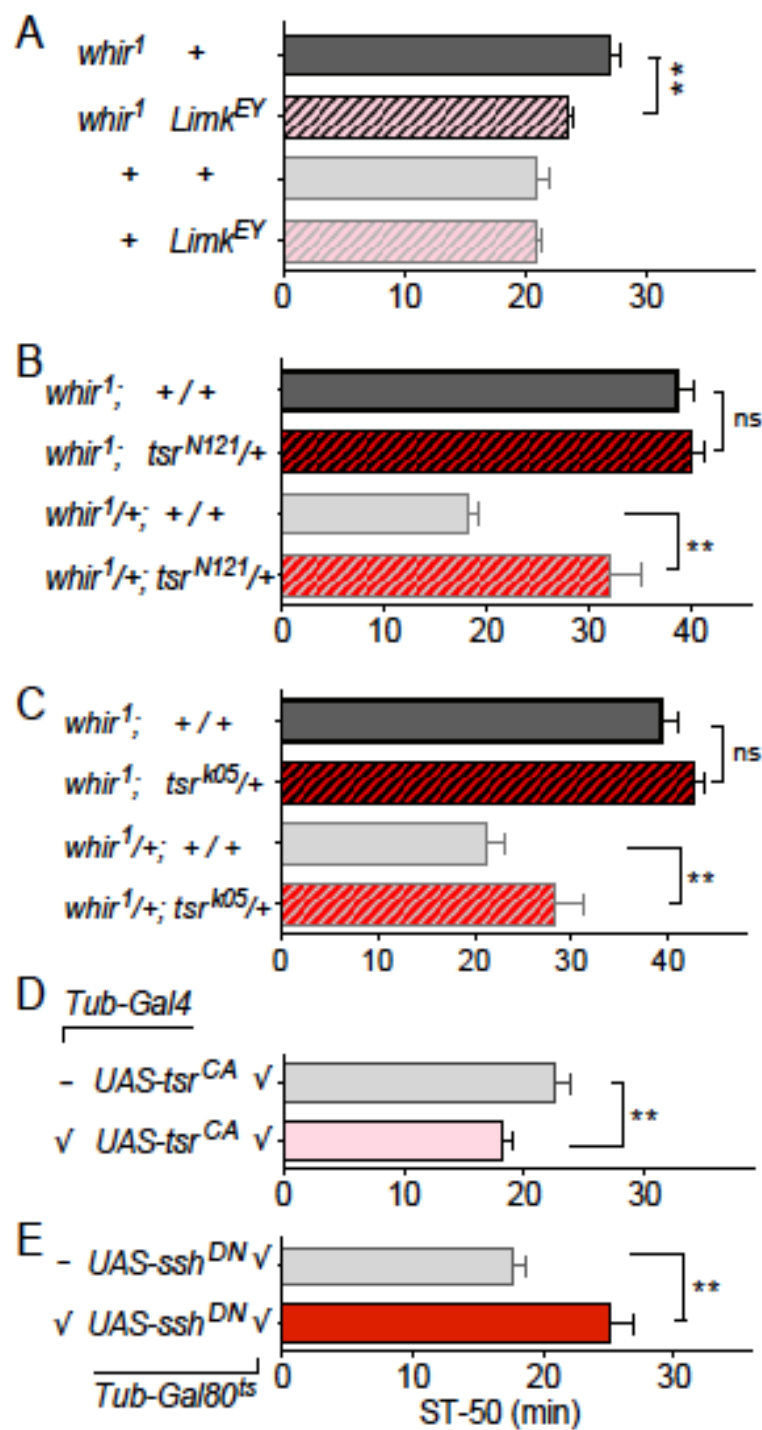
Figure 6.6. Cofilin modulates ethanol-induced sedation *in vivo*. In these graphs, bars represent means ± SEM of time to 50% sedation (ST-50). Flies were exposed to 130/20 ethanol/air flow rate. (A) Loss of function *Limk* mutation has no effect on ethanol-induced sedation on its own, but suppresses *whir*[1] ethanol resistance. (B and C) In phenotypically wild-type whir1/+ flies (Rothenfluh et al., 2006), cofilin loss of function alleles (encoded by the *twinstar*, *tsr*, gene) lead to ethanol-resistance when heterozygous (homozygotes are lethal). Ethanol-resistant *whir*[1] flies are not made more resistant by the introduction of *tsr* loss-of-function mutations, indicating a genetic interaction between *tsr* and *whir*, and suggesting a ceiling effect. (D) Adult-specific expression of constitutively active, un-phosphorylated cofilin causes ethanol sensitivity. (E) Adult-specific expression of a dominant-negative version of cofilin phosphatase (encoded by *slingshot, ssh*) causes ethanol resistance. In (D and E), flies were reared at 18° throughout development to suppress UAS-transgene expression via Tubulin-Gal80[ts] and were then shifted to 29° for 3 days as adults. Student's t-test for significant differences vs. controls ($n \geq 6$, **$p < 0.01$).

CHAPTER 7: Long-lasting, experience-dependent alcohol preference in
Drosophila

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Introduction

The CAFÉ assay represents a major advance in modeling addiction-like behaviors in flies, but it is associated with significant amounts of hands-on time for each genotype assayed. This is a considerable drawback for the implementation of high-throughput screens, which have been a hallmark of Drosophila research (Bellen, Tong and Tsuda, 2010). To improve on the workload associated with the CAFÉ assay, we have developed a novel ethanol consumption assay we have termed FRAPPÉ, for fluorometric reading assay of preference primed by ethanol. The cornerstone of this new assay is the precise reading of volumes ingested from two food solutions labeled with fluorophores, allowing sensitive measurement of less than 5 nl ingested and parametric determination of a preference index (PI) in individual flies. Using the FRAPPÉ, we show that in an acute choice between sucrose with or without 15% ethanol, naïve flies do not show preference. Following various ethanol vapor preexposures, however, they do display significant ethanol consumption preference. The preference induced by an ethanol pre-exposure is long lasting and can also be obtained by ethanol pre-feeding,

even when ethanol is provided as a choice, and not as the sole food source. Lastly, we show that the consumption preference for ethanol induced by a pre-exposure cannot be accounted for solely by the caloric content of ethanol, suggesting a pharmacodynamic action of the drug on the central nervous system. The FRAPPÉ thus represents a novel, high-throughput ethanol preference assay that models numerous aspects of human addiction, including a triggering experience, long-lasting persistence and voluntary ingestion of the drug.

Results

Flies show experience-dependent ethanol consumption preference

In the two-bottle CAFÉ assay, flies are allowed to choose between two food/yeast extract mixtures, one of which contains an addition of 15% ethanol. They are then observed over the span of about 5 days, and consumption preference is measured for the ethanol-containing food mix. In two reports, preference for the ethanol-containing mix seemed to increase slightly over the 5 days assayed (Devineni & Heberlein 2009; Pohl et al. 2012). We wondered whether this increase reflected experience-dependent development of ethanol preference. The CAFÉ assay is based on one reading of consumption per day, thus it is difficult to know whether the first day's reading should be considered coming from naïve flies, or whether one day constitutes significant ethanol-drinking experience. To directly test whether inexperienced, ethanol-naïve Drosophila showed innate ethanol consumption preference, or whether prior experience was necessary for the development of preference, we established a new consumption assay based on fluorometric readings of food volumes consumed by individual flies. In this

assay, termed FRAPPÉ (Fig. 7.1), 30 flies were allowed 10–15 minutes access to a 60-well plate containing 60 mM sucrose with or without 15% ethanol. Significant consumption of at least 10 nl per fly was ensured with a prior period of food deprivation (see Materials and Methods for more details). To determine if ethanol preference was experience dependent, we preexposed the flies to increasing doses of vaporized ethanol the day before the consumption choice. Figure 7.1c shows that naïve flies exposed to air (zero parts ethanol) showed a mild aversion to 15% ethanol (PI < 0) the day following the 20-minute mock exposure. This aversion gradually switched over to preference, as the ethanol pre-exposure increased to 80/70 E/A. At higher doses, flies showed preference indistinguishable from 80/70 pre-exposed flies. These results suggest that ethanol consumption preference in Drosophila is experience dependent.

Next, we sought to investigate which ethanol concentrations pre-exposed flies would prefer to consume. Naïve, unexposed flies showed aversion to ≥ 15% ethanol (Fig. 7.2a), while flies pre-exposed to 80/70 or 150/0 E/A mixtures for 20 minutes the day before showed ethanol preference at ≥10% ethanol (Fig. 7.2b,c). These combined results for both ethanol-exposed groups suggests that pre-exposure to a threshold level of ethanol results in a preference that generalizes across various ethanol concentrations.

Experience-dependent ethanol preference is long lasting

We next sought to investigate the longevity of the ethanol consumption preference induced by a prior ethanol exposure. We exposed flies to a single, 20

minutes dose of 80/70 E/A, or 'mock-exposed' them to a dose of 0/150, and assayed ethanol preference 3, 5, or 8 days later. Flies that were given a one-time pre-exposure showed stable ethanol preference that was observable 3, 5 and even 8 days following exposure (Fig. 7.3b). Mock-exposed flies did not show a preference 8 days after mock exposure, suggesting that preference is experience-dependent and long lasting, and not a function of the flies' age.

Behavioral correlates with ethanol consumption preference

To study a possible relationship between sedation during pre-exposure and subsequent consumption preference, we exposed flies for 20 minutes to E/A pressures ranging from 0/150 to 150/0 E/A and determined the percentage of flies sedated by that exposure. We then assessed ethanol preference the following day. Figure 7.4a shows that sedation steadily increased as a function of the E/A exposure pressure, but preference reached a plateau at 80/70 E/A. While 80/70 and 150/0 E/A preexposure caused the same level of ethanol consumption preference, less than half the flies were sedated after a 80/70 pre-exposure, compared to 100% after 150/0 (Fig. 7.4a). These data suggest that sedation during the pre-exposure is neither necessary for, nor detrimental to subsequent ethanol-preference. Note that flies preexposed from 80/70 to 150/0 all undergo a phase of ethanol-induced hyperactivity prior to sedation. In this experiment, we therefore have not isolated sedation as the sole behaviorally relevant experience that the flies undergo.

To test whether ethanol consumption preference correlates with sedation-tolerance

(Scholz et al. 2000) at the time of consumption choice, groups of flies were preexposed to different E/A pressures and on the next day their ST-50 (time until 50% of flies sedate) to a 110/40 E/A challenge dose was determined. Only the 150/0 preexposure caused measurable sedation tolerance 24 hours later when compared to mock-exposed flies (Fig. 7.4b). Since lower doses of pre-exposure cause consumption preference, but not sedation tolerance one day later, we conclude that tolerance to the sedating effects of ethanol at the time of consumption is not required for ethanol-induced consumption preference.

Different routes of pre-exposure induce consumption preference

In the experiments outlined above, the preference inducing pre-exposure was vaporized ethanol, which the flies passively, and involuntarily breathed. While vaporized ethanol can cause dependence, lead to withdrawal (Goldstein & Pal, 1971) and also to increased consumption in rodents (Roberts et al. 2000), we still wished to investigate whether a more voluntary route of preexposure to ethanol might induce consumption preference. To test this, we first allowed flies ad libitum access to liquid food (sucrose/yeast extract) and water in a CAFÉ-like feeding chamber. After 3 days of feeding in these conditions, flies did not develop ethanol consumption preference in the FRAPPÉ assay (Fig. 7.5b). Second, when we changed the pre-feeding solution to sucrose/yeast extract/15% ethanol, that is the only food available to the flies for the 3 days of pre-feeding contained ethanol, they did develop ethanol consumption preference in the FRAPPÉ choice assay (Fig. 7.5b). Third, we offered flies both food

solutions simultaneously, one of them containing 15% ethanol, thereby giving the flies the choice to consume ethanol or not for the 3 days of pre-feeding. After this choice, flies also developed ethanol consumption preference (Fig. 7.5c). We thus show that multiple routes of ethanol exposure are capable of inducing ethanol consumption preference, including voluntary ethanol consumption itself.

Experience-dependent ethanol preference is independent of caloric content

Even though naïve flies showed slight aversion, or no preference for ethanol (Fig. 7.2a), they might develop a 'taste for' ethanol because of its high caloric content, especially in the voluntary setting of the CAFÉ assay (as suggested by Pohl et al. 2012). It seems less likely that passive exposure to ethanol vapor exposure could be reinforcing for its caloric value. Nevertheless, we decided to directly test the effect of ethanol calories in our consumption preference.

To do this, we offered pre-exposed flies a choice of isocaloric solutions, one containing ethanol, and the other one containing sorbitol, which is tasteless to flies but provides calories (Burke & Waddell 2011; Fujita & Tanimura 2011; Stafford et al. 2012). Mock-exposed flies showed no preference for either sucrose/ethanol versus sucrose/sorbitol, whereas flies that were pre-exposed to 80/70 E/A the previous day showed strong ethanol consumption preference (Fig. 7.6a). Both pre- and mock-exposed flies were able to detect the calories provided by sorbitol, and preferred to drink from sucrose with sorbitol, as opposed to sucrose alone solution (Fig. 7.6b). This suggests that ethanol pre-exposure induced ethanol consumption preference, but it did

not alter pre-existing sorbitol preference. Since naïve flies showed no ethanol preference (see Fig. 7.2a), but did show sorbitol preference (Fig. 7.6b), it was a bit surprising that they only showed a trend toward sorbitol preference when offered together with ethanol (Fig. 7.6a). This would suggest some interaction of ethanol and sorbitol for flies' choice, the nature of which is not clear. To directly test whether ethanol contains metabolically useful calories, we performed an experiment in which we compared the survival of flies maintained on isocaloric sucrose, sorbitol or ethanol. Flies maintained on 1.7 M sorbitol as their only food source showed 12% death after 8 days, which was not significantly different from the 5% death observed in flies kept on isocaloric 600 mM sucrose. Conversely, flies on isocaloric 15% ethanol all died within 5 days (Fig 7.6c). To make sure that there was no confounding interaction of ethanol or sorbitol with sucrose (which was used at 60 mM in Figs 7.6a,b), we repeated the experiment by adding 60 mM sucrose to ethanol and sorbitol. This prolonged survival on ethanol with 60 mM sucrose, compared to ethanol alone, and after 8 days 17% of flies were still alive. However, this was still significantly less survival than flies on sorbitol with 60 mM sucrose showed (88% survival after 8 days), arguing that sorbitol provides significantly more metabolically useful calories to flies than ethanol. The data in Fig. 7.6d also show that flies on ethanol and 60 mM sucrose survived longer than on ethanol alone (suggesting that even 60 mM sucrose provides significant calories to extend survival), but that survival on ethanol with 60 mM sucrose was no different from survival on 60 mM sucrose alone. Together, these data indicate that 15% ethanol is neither toxic, nor caloric to flies, and they are very similar to what has previously been shown by

Xu et al. (2012). Ethanol consumption preference is therefore not driven by caloric value of ethanol, but more likely by the drug's pharmacodynamic properties.

Discussion

In this paper, we describe a novel assay, termed FRAPPÉ, to measure experience-dependent ethanol consumption preference in Drosophila. Up to now, consumption preference in flies has been measured with the CAFÉ assay, a two-bottle choice paradigm that has limited throughput (Devineni & Heberlein 2009). Since flies have been a long-standing model organism for genetic screens (Bellen et al. 2010; Rodan & Rothenfluh 2010), we sought to improve on this limitation and have developed a novel assay that measures the consumption preference index of individual flies in a fluorescence plate reader, thus enabling fast screening of large numbers of flies. Two types of assays in Drosophila model features of addiction most closely: First, the CAFÉ assay measures consumption preference for an ethanol-containing food in groups of (Devineni & Heberlein 2009; Pohl et al. 2012), or even individual flies (Xu et al. 2012). In this assay, the flies' preference tends to increase over the course of the 5-day experiment. When consumption is followed by a period of 3 days of forced abstinence, the flies immediately return to their acquired high preference right away, suggestive of relapse-like behavior (Devineni & Heberlein 2009). Furthermore, flies will acquire preference even for a bitter-tasting quinine solution, which is normally aversive, when added to the ethanol/food mixture, arguing that they are motivated to overcome aversion in order to consume ethanol-containing food (Devineni & Heberlein 2009). The second behavioral paradigm developed in flies is similar to conditioned place preference used

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with rodents, only that in the case of flies, the stimulus reinforced with ethanol vapor is an innocuous odor (Kaun et al. 2011). After conditioning, flies prefer the ethanol-paired odor, and they are willing to cross an aversive foot-shock grid to approach that odor. Interestingly, ethanol seems to be a stronger reinforce than sucrose, which is generally used in appetitive conditioning (Kaun et al. 2011). Our novel FRAPPÉ assay combines the experience-dependent aspect of the odor-conditioning paradigm with the voluntary ethanol consumption of the CAFÉ and allows for the high-throughput measure of ethanol preference in individual flies.

What kind of ethanol-experiences cause consumption preference in the FRAPPÉ? We show that pre-exposures to ethanol vapor that induces consumption preference include hyperactivating doses, as well as sedating doses (see Fig. 7.4a). The dose response of the consumption PI as a function of the pre-exposure dose shows a steady increase at low to medium doses, but then reaches a plateau at high doses (Fig. 7.1c). This plateau contrasts with ethanol-conditioned odor preference, where only hyperactivating, but not sedating doses of ethanol vapor induced subsequent odor preference (Kaun et al. 2011). This difference could have numerous causes: First, it could reflect a mechanistic difference between the FRAPPÉ and conditioned odor preference. Second, neuronal cell death in the antenna (French & Heberlein, 2009)— the major olfactory sensory organ in flies— caused by the sedating conditioning dose of ethanol vapor could confound subsequent odor choices. Third, some of our pre-exposure regimens also might lead to ethanol aversion in the short term, followed by long-term ethanol preference. We have not tested flies in the FRAPPÉ assays within a

few hours after the preexposure, but Kaun et al. (2011) found that even for reinforcing ethanol exposures, flies initially developed odor avoidance, which only later, after 12–15 hours changed into odor preference. Our finding that even a sedating dose of ethanol can increase subsequent ethanol preference and consumption is not unprecedented. In rats, a single motor impairing dose of intraperitoneal ethanol injection increased their subsequent ethanol intake compared to saline-injected rats (Tampier & Quintanilla 2002). Ethanol-induced sedation causes stress and induces expression of numerous stress-related genes (Kong et al. 2010). It seems unlikely that a generalized stress response to the pre-exposure is causing subsequent ethanol-preference in our FRAPPÉ assay. First, flies are food deprived for 18 hours prior to consumption choice, and this stress was insufficient to induce preference in naïve flies (Figs 7.1c & 7.2a). Second, two other stressors, 6-hour dehydration and repeated mechanical stress by vortexing 24 hours before the choice assay, also did not lead to consumption preference (data not shown). Lastly, we found that ethanol consumption itself in a CAFÉ-like choice setting for 3 days was capable of causing subsequent ethanol consumption preference in the FRAPPÉ. Different ways of ethanol pre-exposure can thus lead to consumption preference, including voluntary consumption, which is unlikely to be stressful. Our 3-day pre-feeding data, together with the gradual development of ethanol preference in the CAFÉ assay mentioned earlier, would indicate that ethanol-experience causes a gradual shift from slight aversion/ indifference, toward preference. While non-tasty, but caloric foods can act as reinforcers within minutes (via unknown sensory mechanisms; Burke & Waddell 2011; Fujita & Tanimura 2011), the 15-minute acute ethanol

preference choice in the FRAPPÉ assay does not appear to be long enough for flies to 'learn' to prefer ethanol. Since ethanol is not of caloric value to flies (see below), it presumably acts via a different reinforcing pathway that is yet to be determined. In line with this gradual learning of the reinforcing aspects of ethanol are the findings by Kaun et al. (2011) who showed that ethanol-conditioned odor preference only developed 12–15 hours after the ethanol conditioning. The kinetics of ethanol-mediated odor conditioning, and preference development thus seem slower than those observed in classical appetitive conditioning (Burke &Waddell 2011; Fujita & Tanimura 2011). Despite these differences in the kinetics of behavioral changes, it is noteworthy that the two mutations affecting ethanol consumption in the CAFÉ assay, *kra* (Devineni & Heberlein 2009) and *rut* (Xu et al. 2012), were both initially isolated and described as associative learning and memory mutants in Drosophila. Development of ethanol preference in flies may thus share molecular mechanisms that are also utilized for long-term learning and memory formation (Rothenfluh & Cowan 2013), in line with current thinking of addiction as long-lasting, maladaptive reinforcement learning (Grueter, Rothwell & Malenka 2012). Both experience-dependent consumption preference in the FRAPPÉ, as well as ethanol-conditioned odor preference are long lasting and are still present even a week after the last ethanol experience (Fig. 7.3; Kaun et al. 2011). Further support for shared mechanisms between these ethanol-induced behavioral changes and other associative learning and memory processes comes from a survey of over 60 Drosophila learning and memory mutants. The results showed a striking overrepresentation of phenotypes in ethanol-induced behaviors, including tolerance

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(Berger et al. 2008).

In another small survey of behavioral ethanol mutants, Devineni et al. (2011) found that consumption preference phenotypes in the CAFÉ assay correlated with rapid tolerance phenotypes, but not with ethanol-induced sedation, or hyperactivity phenotypes. Our results (Figs. 7.4 & 7.5) indicate that sedation during the preexposure is neither necessary for, nor detrimental to preference induction. In addition, flies need not be tolerant to ethanol-induced sedation at the time of consumption choice. Furthermore, while flies can become hyperactive after ethanol ingestion, they do not routinely seem to in the CAFÉ (Devineni & Heberlein 2009), or CAFÉ-like prefeeding assay (Fig. 7.5). This raises the questions what does ethanol do to flies to induce preference, and why do flies consume alcohol in the first place. Two obvious answers spring to mind. First, they 'like' the pharmacodynamics effects that ethanol has on the brain. This is what causes people to drink and abuse alcoholic beverages. Second, flies might prefer ethanol-containing food for the considerable calories that are provided by ethanol. Indeed, in our experiments, the difference between the food with and without ethanol was 907 versus 81 mcal/µl. One report altered this imbalance by varying sucrose, but not ethanol concentration in the CAFÉ and found no change in PI for ethanol (Devineni & Heberlein 2009), arguing against caloric imbalance being the driving force for preference. Xu et al. (2012) altered sucrose concentration and found a resulting change in volume (but not calories) consumed. Changes in ethanol concentration led to no change in volume, but in total calories consumed (including ethanol's). This argues that sucrose, but not ethanol consumption, is under homeostatic caloric control. In contrast,

a recent paper investigated preference in the CAFÉ after counterbalancing ethanol's calories with the same amount of calories in the other capillary. The complex sugar maltodextrin, and both sucrose and glucose, but not mannose, abrogated preference for the ethanol-containing capillary (Pohl et al. 2012), suggesting that it is the caloric content of ethanol that drives the preference. Alternatively, preference for glucose and sucrose could be due to their highly appetitive sweet taste (Pohl et al. 2012), since taste is a major driving force in food choice (Stafford et al. 2012). Ethanol, in contrast, does not have any taste at the doses offered (Devineni & Heberlein 2009; Pohl et al. 2012). The remaining results with tasteless, but caloric maltodextrin and mannose were equivocal (Pohl et al. 2012). We decided to steer clear of maltodexrin, as it can be contaminated by simple and tasty sugars (Burke & Waddell 2011), and instead used sorbitol to counterbalance ethanol's calories. Sorbitol is a tasteless, caloric sugar (Burke & Waddell 2011; Fujita & Tanimura 2011; Stafford et al. 2012). Our results show that ethanol-exposed flies preferred to consume ethanol-containing food over isocaloric sorbitol-containing food, while we observed no preference in unexposed flies. When offered sucrose versus sucrose with sorbitol, flies strongly preferred the sorbitol-containing solution, irrespective of prior ethanol experience. These experiments argue that (1) ethanol provides an appetitive force that cannot solely be accounted for by the calories it contains, (2) even in our relatively acute FRAPPÉ paradigm, where flies choose for only 15 minutes, flies can detect, and prefer the caloric content of a taste-neutral sorbitol solution, and (3) ethanol preexposure does not affect the perception of tasteless calories. Taken together, the above experiments, with the one exception of the

maltodextrin experiments, argue that ethanol does not provide significant calories as a driving force for preference. Indeed, when we tested survival of flies on 15% ethanol compared to 'isocaloric' sucrose or sorbitol, flies survived significantly longer on the sugars compared to ethanol (Fig. 7.6c,d), arguing that the theoretical calories provided by ethanol are not efficiently utilized by flies' metabolism, and that flies prefer to consume ethanol for its pharmacodynamic effects. One confound of our FRAPPÉ assay is the need for food deprivation, in order to ensure significant amounts of consumption. While starvation in and of itself did not cause ethanol consumption preference, it is possible that it is a necessary gating mechanism for preference to be expressed. The similarities we have observed between the CAFÉ and the FRAPPÉ assays would argue against that, given that flies are not food deprived in the CAFÉ assay. We are currently working on FRAPPÉ approaches that do not rely on food deprivation, to address this issue. Nevertheless, we have developed a novel experience-dependent ethanol consumption assay, which induces long-lasting ethanol consumption preference. Since this assay is quick, but allows for precise measurement of an individual preference index, it can be used in large-scale genetic screens to investigate the molecular mechanisms that are involved in the development of experience-dependent drinking, and should further our understanding of the processes leading to alcohol addiction.

Utilizing this modified Café paradigm, I will explore what structures and circuits in the fly brain are required to influence the aversive and appetitive properties of alcohol. In the next chapter, I will first review and discuss the fly brain structures and how they have been implicated in learning and reward.

Experimental methods

Flies

Behavioral experiments were performed with male w^{1118} Berlin flies, which were raised at 25°C and 70% humidity on standard cornmeal/molasses food. Flies were 1–5 days of age at the start of the experiments.

Booz-o-mat exposure

The day before ethanol vapor exposure, male flies were collected in groups of 30 and put on unyeasted food. The following day, flies were transferred into the booz-o-mat apparatus for a 20-minute exposure at desired ethanol to air ratio (E/A) as described (Wolf et al. 2002). Flies were placed back into unyeasted food vials for 4 hours to recover, and were then transferred to vials filled with 0.7% agar solution (for hydration). These vials were placed into a 25°C/70% humidity incubator for an 18-hour food deprivation.

Ethanol consumption preference

All ethanol preference experiments were conducted with a 10–15-minute choice of 60 mM sucrose with 15% ethanol versus 60 mM sucrose unless otherwise stated (Fig. 7.2, and Fig. 7.6a,b). Access duration was limited to prevent dye loss via excretion associated with longer feeding times (data not shown). Because flies drink in long, uninterrupted bouts, with little well-to-well movement, if presented with high-sucrose foods (Penninti & Rothenfluh, unpublished), the sucrose concentration was kept low to

increase sampling from different wells and to increase the probability that concentrations consumed reflected a true preference of individual flies. This in turn required that flies were food-deprived for 14–18 hours, to ensure large enough quantities of ingestion for accurate FRAPPÉ measurements. Using fluorescent dyes, 0.005% rhodamine B (Acros Organics, Pittsburgh, PA, USA) and 0.003% fluorescein sodium salt (Sigma-Aldrich Co., St. Louis, MO, USA), color counter-balanced solutions were made. In one plate, the ethanol containing solution was colored with rhodamine B, and the sucrose-only solution with fluorescein, with opposite color pairing in the counter-balanced one. This eliminated potential color bias in the preference assay. After a 10–15-minute feeding period, flies were placed in 15 ml Falcon tubes, frozen in dry ice and vortexed to shear legs, heads and wings from the torsos/abdomen body core. The cores were then individually placed into the wells of a 96-well flat-bottom tissue culture plate containing 50 µl of water to keep flies centered in the excitation beam, and to increase the likelihood of their ventral side facing up; ventral being the side with the least dark cuticle, thus minimizing absorption of fluorophore emission. These whole fly core measurements correlated well with readings from flies after homogenization, centrifugation and supernatant reading in the Fluoroskan (Supporting Information Fig. 7.7). Using Ascent Software v2.6, fluorescence data was then collected in a Fluoroskan Ascent FL2.4 plate reader (Thermo Scientific, Pittsburgh, PA, USA) for rhodamine B at excitation/emission wavelengths of 542/591 nm and at 485/527 nm for fluorescein. After taking five separate measurements, which were each followed by a 'shake' step (to maximize the chance of the ventral side of fly cores facing up, thus minimizing cuticle

absorption, see Supporting Information Fig. 7.8), the maximum fluorescence values were recorded for each well. Rhodamine B values were multiplied by 3.23 to convert to nl consumed, while the maximum fluorescein values were multiplied by 1.74, with both conversion factors empirically determined using correlations with NanoDrop spectrophotometer (Thermo Scientific) readings. Overall, our individual fly Fluoroskan readings agree well with individual readings from flies fed FD&C Blue #1 (Sigma-Aldrich Co.) and measured for volumes ingested in a NanoDrop photospectrometer (Supporting Information Fig. 7.8). The sensitivity of this fluorometric assay is better than 3 nl, but we excluded flies drinking less than 10 nl to increase the likelihood of ingestion from both solutions. A PI for each individual fly was calculated (nl consumed of sucrose/ethanol— nl consumed of sucrose)/(nl consumed of sucrose/ethanol + nl consumed of sucrose), ranging from +1 = total preference, to −1 = total avoidance.

Ethanol sedation/tolerance

Ethanol sedation and tolerance experiments on groups of 20 flies were carried out using the loss-of-righting reflex (LORR) test as described previously (Rothenfluh et al. 2006). For tolerance, flies pre-exposed 24 hours prior, were subjected to a 110/40 E/A challenge dose, and the time to 50% sedation/LORR (ST-50) determined.

Feeding experiments

Flies were pre-fed in a modified CAFÉ assay over a span of 3 days in rectangular 4-well plates (127.8 Å~ 85.5 mm, Thermo Scientific; Fig. 7.5). Food was provided in 0.2

ml tubes with a 27 G needle hole at the bottom for drinking access, a 27 G hole atop for pressure equilibration and a 25 G hole atop for filling with solution. Fifty flies per well had access to two food tubes (5% sucrose/5% powdered yeast extract) and two water tubes. For some groups, the food solution was supplemented with 15% ethanol. After 3 days of feeding, flies were food deprived on 0.7% agar for 14 hours, and ethanol-consumption preference measured as described above. The same setup was utilized to determine survival on different carbohydrates (Fig. 7.6c). Wells included a piece of 3 mm filter paper for contrast making dead flies easily visible. Calories were balanced in the different wells calculating from 4 kcal/g (sucrose), 7 kcal/g (ethanol) and 2.6 kcal/g (sorbitol).

Statistics

Statistics were calculated using Prism5 for Mac OSX (Graph Pad Software, La Jolla, CA, USA). Preference indices for a given group were not distributed normally (D'Agostino/Pearson omnibus normality test), and were plotted as medians with quartile boxes and 10–90% whiskers. They were tested for preference/avoidance (i.e. PI <> 0) using the Wilcoxon signed-rank test. The legend in each figure of PIs includes the median, number of flies, P value for preference (PI <> 0), and the Wilcoxon signed-rank test sum for each group. Whenever no preference/avoidance was found, we made sure that at least an n of 50 flies was assayed, for adequate statistical power. Differences between PIs were queried using the Mann–Whitney U-test.
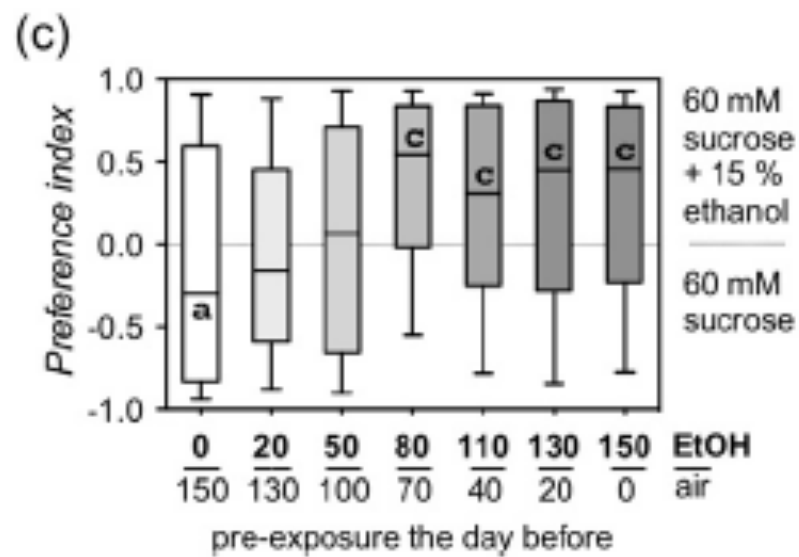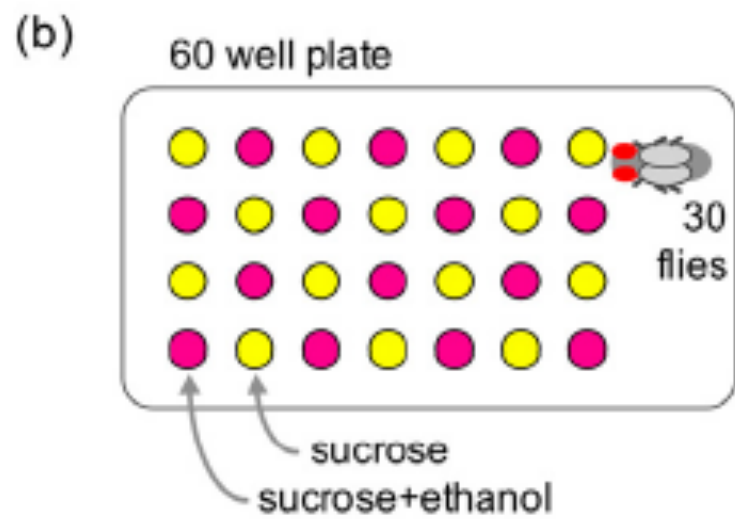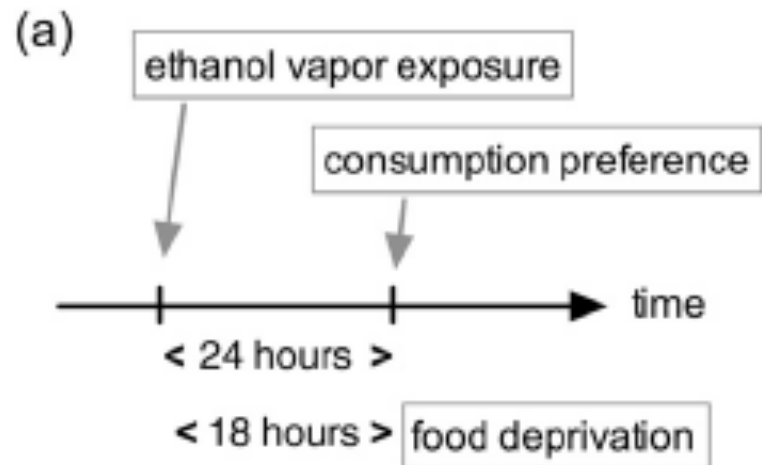
(a) ethanol vapor exposure → consumption preference
< 24 hours >
< 18 hours > food deprivation

(b) 60 well plate
30 flies
sucrose
sucrose+ethanol

(c)
60 mM sucrose + 15 % ethanol
60 mM sucrose
pre-exposure the day before

Figure 7.1. The FRAPPÉ, an experience-dependent ethanol consumption preference assay in Drosophila. (a) Schematic of the experimental design. (b) Schematic of the consumption plate, where flies chose between 60mM sucrose and 60mM sucrose + 15% ethanol after 14–18 hours of food deprivation. The food is labeled with (counterbalanced) fluorescent dyes. (c) Preference index of flies pre-exposed the day before for 20 minutes to the indicated vaporized ethanol/air (E/A) pressures. Data shown here, as in subsequent preference graphs, are medians, with quartile bars, and 10–90th percentile whiskers. Mock exposed flies (i.e.no ethanol) show mild aversion (PI < 0) to the ethanol-containing food the day after exposure, while flies exposed to 80/70 E/A or higher show significant preference (for this, and following preference graphs, a: P < 0.05 of the indicated group being different from 0, Wilcoxon Signed Rank Test, b: P < 0.01, c: P < 0.001 for each group, where one fly is an n of one.) Statistics for each group from left to right including median, number of flies, P value for preference/ avoidance, Sign Test sum (as in following legends) were as follows for 0: –0.29, 130, 0.03, –18; 20: –0.15, 98, 0.42, –5; 50: 0.07, 90, 0.67, 3; 80: 0.54,144, < 0.001,54; 110:0.31,174,< 0.001,37;130:0.45,223,< 0.001, 58; 150: 0.46, 107, < 0.001, 31

(a)

5%   10%   15%   20%   25%   x %
                              ethanol
                              with

                              60 mM
                              sucrose

                              60 mM
                              sucrose

naïve flies

(b)

5%   10%   15%   20%   25%   x %
                              ethanol
                              with

                              60 mM
                              sucrose

                              60 mM
                              sucrose

pre-exposed to **80/70** the day before

(c)

5%   10%   15%   20%   25%   x %
                              ethanol
                              with

                              60 mM
                              sucrose

                              60 mM
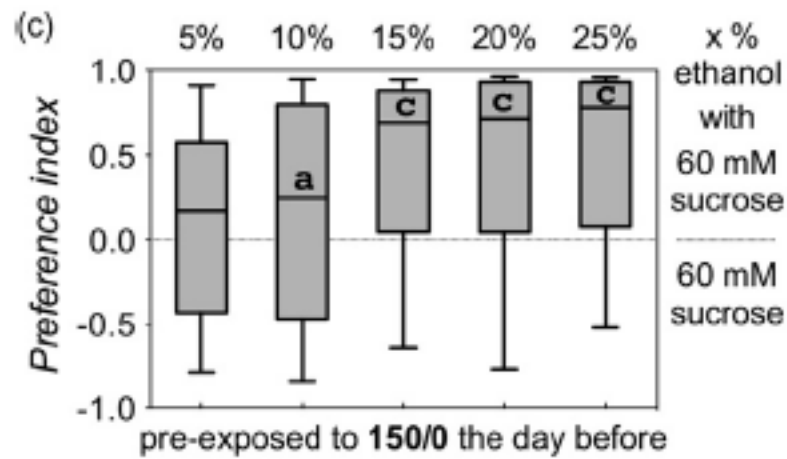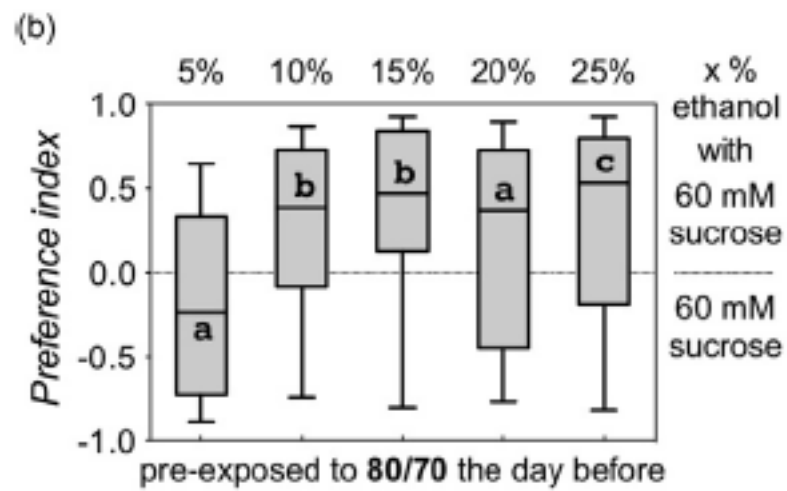   sucrose

pre-exposed to **150/0** the day before
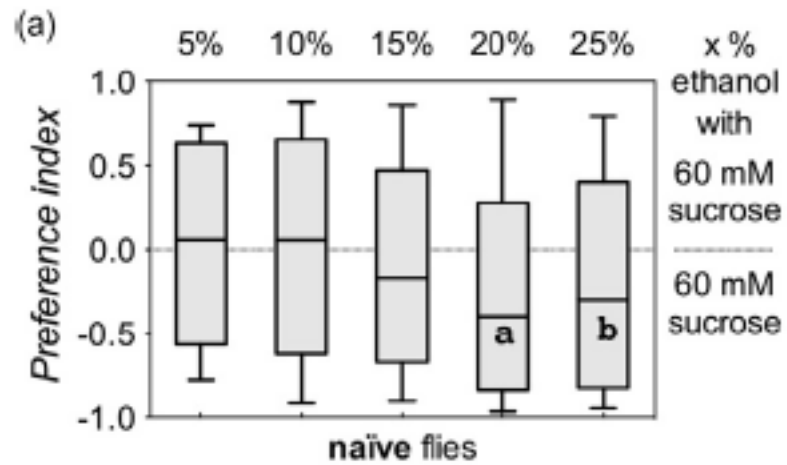
Figure 7.2. Pre-exposed flies tested 24 hours later show ethanol preference to various ethanol concentrations. (a) Naïve flies avoid high (≥15%) ethanol concentrations. Statistics: 5%: 0.06, 49, 0.98, 0; 10%: 0.06, 57, 0.89, 1; 15%: −0.17, 168, 0.07, −15; 20%: −0.40, 43, 0.018, −11; 25%: −0.30, 92, 0.004, −18. (b) Flies pre-exposed to 80/70 E/A show ethanol preference for ≥10% ethanol. Statistics for 5%: −0.24, 79, 0.008, −14; 10%: 0.29, 36, 0.021, 9; 15%: 0.47, 44, 0.003, 15; 20%: 0.37, 73, 0.033, 11; 25%: 0.53, 71, < 0.001, 20. (c) Pre-exposure to a high ethanol dose also causes ethanol preference for ≥10% ethanol. Statistics for 5%: 0.17, 93, 0.14, 8; 10%: 0.24, 88, 0.011, 16; 15%: 0.69, 144, < 0.001, 61; 20%: 0.72, 148, < 0.001, 62; 25%: 0.78, 120, < 0.001, 57
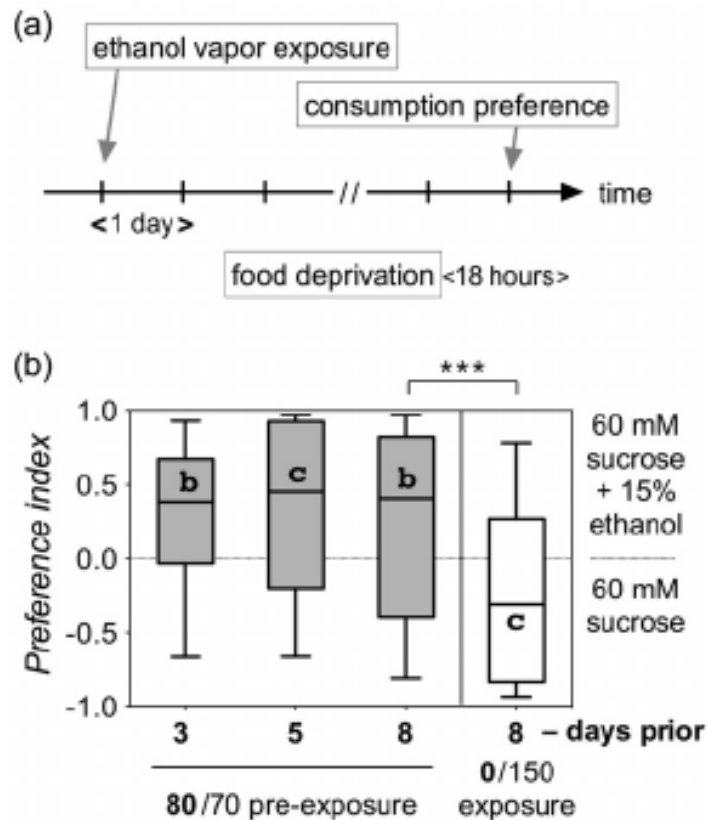
Figure 7.3. Ethanol pre-exposure causes long-lasting preference. (a) Experimental design. Flies were exposed to one dose of 80/70 E/A (or 0/150 mock exposed), and then assayed the indicated number of days later for their ethanol consumption preference. (b) Even 8 days after a one-time exposure, flies still show ethanol preference, while mock-exposed flies still avoid ethanol (*** P < 0.001, U = 2056, for 8 days after 80/70 versus 0/150 exposure, Mann–Whitney U-Test). Statistics for 3 days: 0.38, 36, 0.009, 9; 5 days: 0.45, 55, < 0.001, 19; 8 days: 0.40, 67, 0.009, 15; 8 days mock: –0.31, 100, < 0.001, –23

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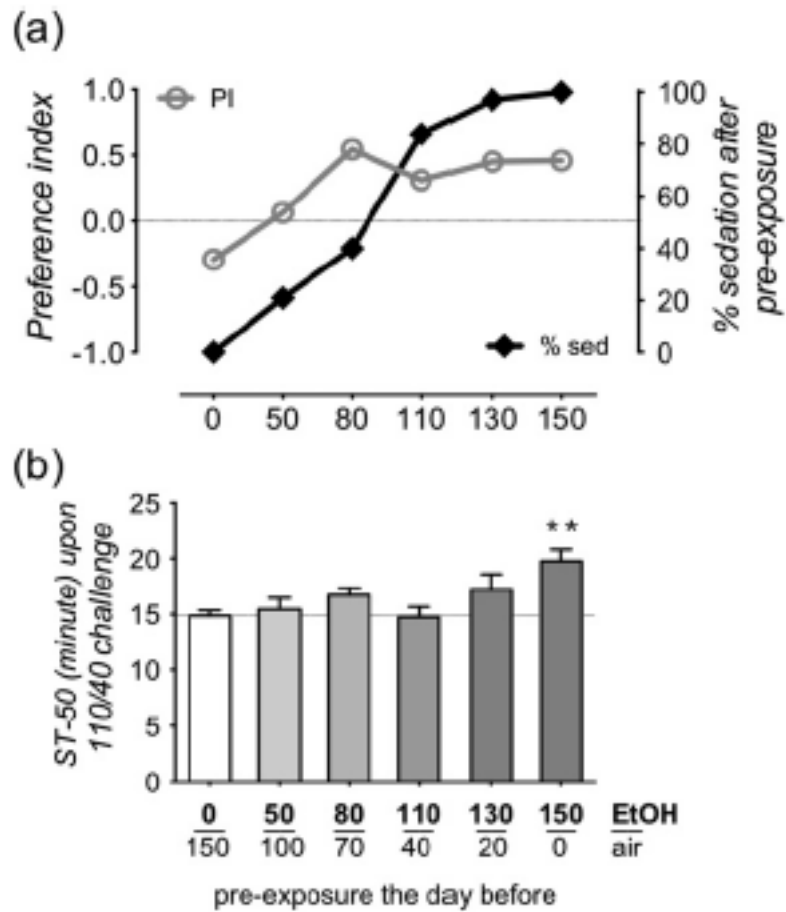
Figure 7.4. Ethanol consumption preference does not require preexposure sedation, or induction of rapid tolerance. Note that the same x-axis applies to both panels. (a) 20-minute pre-exposure leads to levels of sedation (black line, right axis, n ≥ 90 per dose), which steadily increase as a function of the ethanol/air exposure pressure. Ethanol consumption preference 24 hours later reaches a peak at 80/70 (where only 41% of flies sedate) and does not increase further (grey line, left axis, medians re-plotted from Fig. 1c). (b) 24 hours after pre-exposure, a different set of flies shows that only pre-exposure to 150/0 E/A causes increased time to sedation (i.e. tolerance) compared to mock-exposed flies (** $P < 0.01$, q = 3.9, Dunnett's **post hoc** multiple comparison test of all groups versus 0/150 mock exposure. n = 6 groups of 20 flies per group.)
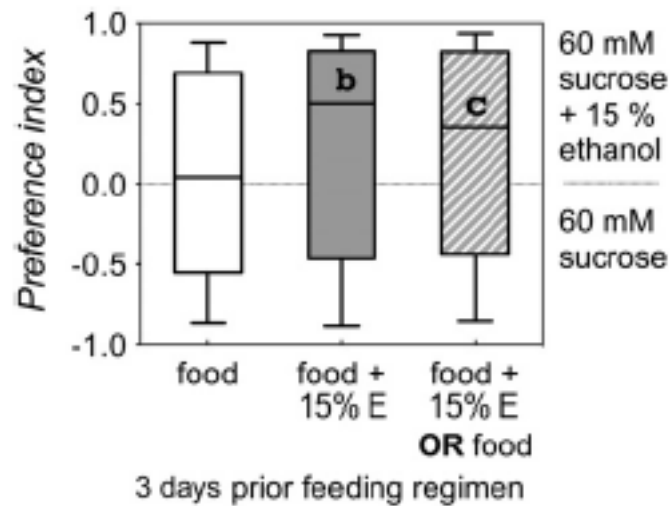
Figure 7.5. Voluntary ethanol consumption induces ethanol preference. Flies were fed for 3 days with either 5% sucrose/5% powdered yeast extract (labeled 'food'), or sucrose/yeast extract/15% ethanol (labeled as 'food + 15% E'), or allowed to choose between those two solutions (labeled as 'food + 15% E OR food'). Flies that had prior access to ethanol developed subsequent consumption preference in the FRAPPÉ, while flies that ate ethanol-less food only did not. Statistics for food: 0.04, 140, 0.43, 5; food + ethanol: 0.50, 100, 0.007, 21; food or food + ethanol: 0.37, 187, < 0.001, 33
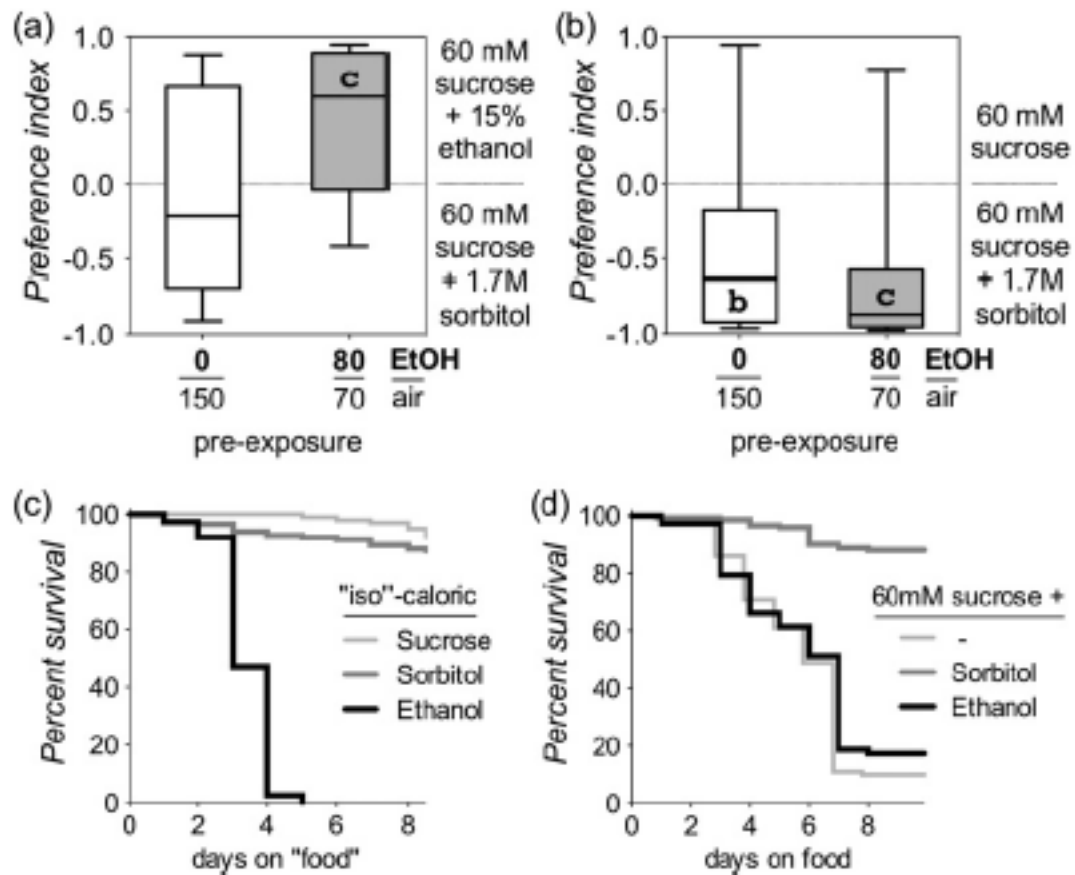
Figure 7.6. Ethanol consumption preference is not driven by ethanol calories. (a) Pre-exposed flies given a choice between sucrose+15% ethanol versus sucrose+1.7M sorbitol (a tasteless but caloric sugar for flies) will prefer ethanol. Note that the two solutions to choose from are nominally isocaloric. Statistics for 0: –0.22, 200, 0.31, –11; 80: 0.60, 253, < 0.001, 102. (b) In the absence of ethanol, flies strongly prefer the sorbitol-containing sucrose. Statistics for 0: –0.64, 48, 0.005, –18; 80: –0.87, 64, < 0.001, –37. (c) Unlike sucrose and sorbitol, ethanol provides minimal calories for survival, and by 4 days on 15% ethanol alone, all flies die. Isocaloric sugars offered in parallel were 600mM sucrose, or 1.7M sorbitol (P < 0.001, chi-square = 210, for ethanol versus sorbitol survival curve, Gehan-Breslow-Wilcoxon Test, n ≥ 149 flies per condition). (d) 15% Ethanol also provided no usable calories when offered together with 60mM sucrose, and flies die as quickly as with 60mM sucrose alone. A 1.7M sorbitol significantly extends the survival when added to 60mM sucrose (P < 0.001, chi-square = 168, for ethanol versus sorbitol survival curve, Gehan-Breslow-Wilcoxon Test, n ≥ 150 flies per condition)

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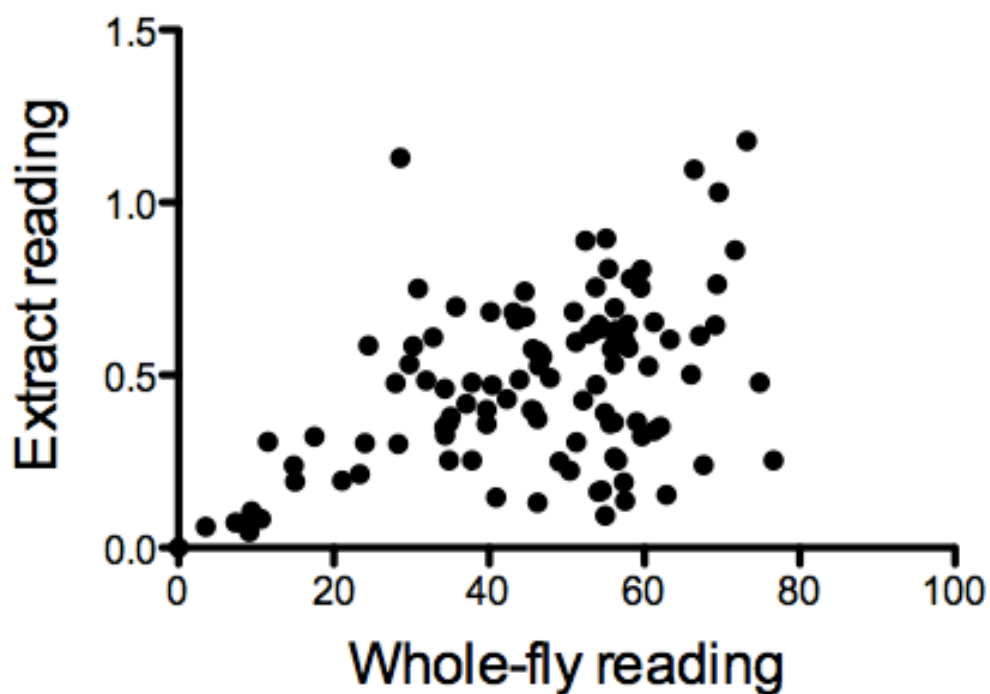
Figure 7.7. Correlation of fluorometric readings from whole fly cores (X-axis) and fly extracts (Y-axis) shows a Pearson's correlation coefficient of 0.48, p<0.001. Individual flies' emissions were first measured from whole cores, and then each core was individually homogenized, centrifuged, and the supernatant put back into the Fluoroskan plate reader.
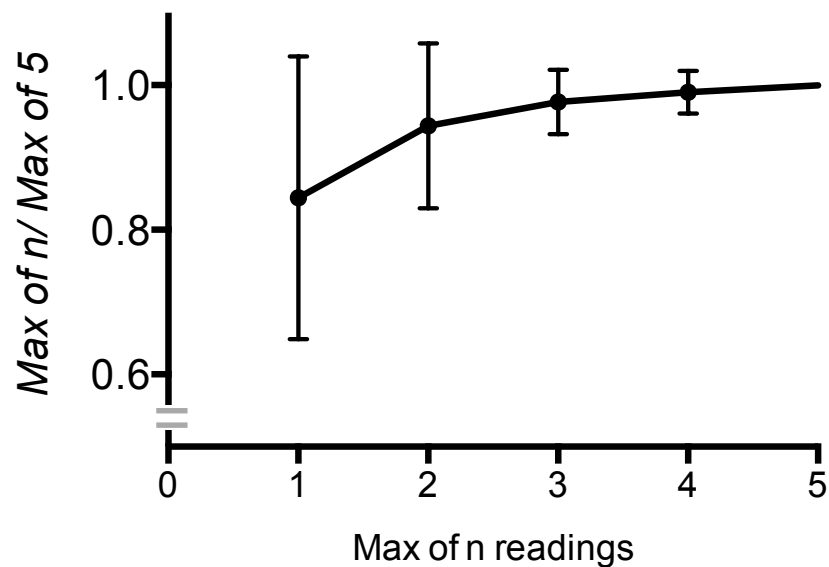
Figure 7.8. Effect of successive shake steps in the Fluoroskan plate reader. The emission from each well was measured, then the plate was shaken, and the next reading was taken. For each successive reading (X-axis), the maximum reading of that well was determined, and normalized to the maximum after 5 readings (Y-axis). The curve shows that after 3 shake steps the readings do not change very much, suggesting that 5 shake steps are an adequate way to get the highest fluorescence reading from each well/fly.
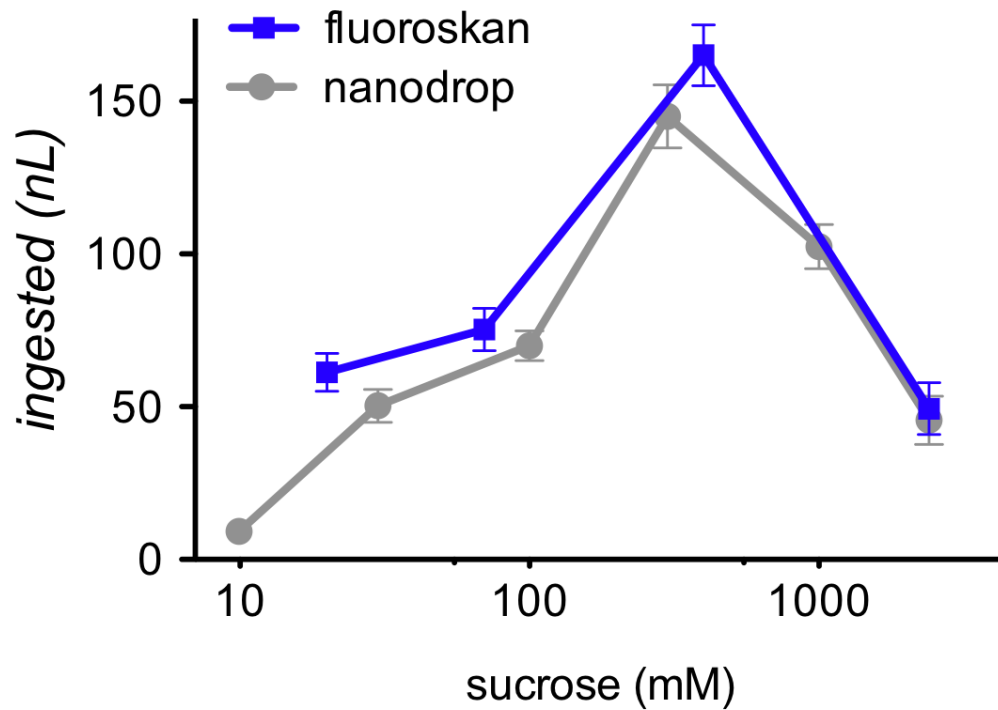
Figure 7.9. Concordance of fluorometric readings with absorption readings from a NanoDrop spectrophotometer. *w Berlin* flies were starved for 18 hours, and then offered various sucrose concentrations (X-axis) supplements with either FD&C Blue #1 (grey curve), or Rhodamine B (blue curve). Blue #1 absorption was measured after homogenization of individual flies, while Rhodamine emission of a different set of individual flies was measured in a Fluoroskan plate reader. The determined ingested volumes are indistinguishable by measurement method, but distinguishable by sucrose concentration offered.

CHAPTER 8: Distinct dopaminergic neuro-circuits mediate Naïve alcohol aversion and Experience-Dependent alcohol preference in *Drosophila*.

* This chapter has been prepared for submission. Aylin R. Rodan, Adrian Rothenfluh, and I designed experiments, analyzed and interpreted data in collaboration with Jill Venton and her lab members. All fly experiments were performed by me, and I wrote the paper while others edited/approved the final version for publication.

Introduction:

Preference for cues associated with drugs of abuse, are observed to spontaneously switch from a negative (aversion) to a positive (attractive/appetitive) valence after hours of conditioning (Kaun et al., 2012; Laviolette and van der Kooy, 2004; Pautassi et al., 2008; Pautassi et al., 2009). These opposing valences may result from conflicting responses to both the sensory and pharmacological effects of addictive drugs (Devineni and Heberlein, 2009; Kaun et al., 2012; Pautassi et al., 2008; Pautassi et al., 2009), and the understanding of how drugs of abuse primarily affect aversive- and rewarding-neural circuits leading to addiction remains incomplete. Like mammals, opposing valences to alcohol's effect can be modeled in the fruit fly, *Drosophila melanogaster*. Flies exhibit naïve alcohol aversion (NAA) when exposed to alcohol or alcohol-associated cues for the first time (Kaun et al., 2012; Peru et al., 2014). However, if exposed to vaporized alcohol 24 hours prior to the choice test (i.e. in a Café assay and an Odor-place preference paradigm), flies show preference for alcohol in a dose dependent manner (Kaun et al., 2012; Peru et al., 2014). Additionally, prior experience of alcohol in flies causes long-lasting effects on behavior with alcohol preference observed for over 7 days (Experience-dependent alcohol preference (EDAP) (Kaun et

al., 2012; Peru et al., 2014). In this study, we investigated whether the neurotransmitter dopamine (DA) is important for NAA or EDAP in flies.

Results

Dopamine expression is required for both naïve alcohol aversion (NAA) and experience-dependent alcohol preference (EDAP) in flies

As described previously (Peru et al., 2014), we show that mock-exposed (0/150 ETOH/Air (E/A)) wild-type flies exhibit NAA in a 16-hour café assay (Ja et al., 2007), but show EDAP if exposed at a 50/100 E/A dose 24 hours prior to a choice between food and ethanol-containing food (Figure 8.1A, B). Using this paradigm, we explored whether the neurotransmitter DA: important for rewarding and motivational valence in mammals (Schultz, 2002), is required for alcohol preference in flies. To explore a role for DA in alcohol preference, we severely reduced DA levels by feeding flies a competitive antagonist of tyrosine hydroxylase (TH) called 3-iodotyrosine (3-IY) (Neckameyer, 1996). TH is a rate-limiting enzyme required for the conversion of tyrosine to L-DOPA, a precursor DA synthesis (Figure 8.1A). Compared to wild-type, flies treated with 3-IY for 48 hours (Bainton et al., 2000; Neckameyer, 1996) and then exposed to 0/150 E/A for 20 minutes (Wolf et al., 2002) show naïve preference for alcohol (Figure 8.1A, 8.1B). On the other hand, naïve flies treated with L-DOPA (Bainton et al., 2000; Neckameyer, 1996), increasing their DA levels, show increased aversion to alcohol when compared to wild type in the café assay (Figure 8.1A). Feeding L-DOPA to flies treated with 3-IY rescues the naïve preference observed in 3-IY treated flies. At a 50/100 E/A dose, flies

with more DA (L-DOPA treated) do not exhibit EDAP, while flies with less DA (3-IY treated) show similar preference as wild type suggesting that dopamine expression is necessary for alcohol aversion in flies (Figure 8.1C). Although surprising, DA has previously been observed to be required for aversion while octopamine, the fly homolog of norepinephrine, is required for appetitive rewards in flies (Schwaerzel et al., 2003). However, recent research shows that distinct dopaminergic neuron clusters are required for both olfactory aversion and appetitive conditioning with the PAM DA (Figure 8.1D) neurons required for odor reward memory while DA neurons expressed by TH-Gal4 (Figure 8.1D) are required for aversive odor memory (Burke et al., 2012). Furthermore, the PAM DA neurons are required for alcohol-associated odor- and oviposition-preference in flies (Azanchi et al., 2013; Kaun et al., 2012). Using the PAM (R58E02)- and TH-Gal4, we therefore investigated whether silencing or activating these distinct DA neurons (Figure 8.1D) affected alcohol preference by using thermo-genetics in our Café paradigm (Hamada et al., 2008; Kitamoto, 2002; Pulver et al., 2009). Using the Gal4-UAS system (Brand and Perrimon, 1993), we either activated or silenced PAM- and TH-Gal4 DA neurons during ethanol exposure and during the choice (Café assay) experiment by expressing UAS-$TrpA^{ts}$ and UAS-Shi^{ts} respectively at 30°C (Figure 8.1E). Activating TH-Gal4 neurons at 30°C (TH-Gal4 UAS-$TrpA^{ts}$) caused alcohol aversion at a 50/100 ETOH/air dose when compared to controls (TH-Gal4 UAS-GFP, Figure 8.1F), while silencing TH-Gal4 neurons (TH-Gal4 UAS-Shi^{ts}) caused naïve alcohol preference at a 0/150 ETOH/air dose (Figure 8.1F) compared to controls. Alternatively, activating PAM-Gal4 neurons at 30°C (PAM-Gal4 UAS-$TrpA^{ts}$, Figure 8.1E), led to alcohol

preference in a dose dependent manner when compared to controls (PAM-Gal4 UAS-*GFP*), while silencing PAM-Gal4 neurons (PAM-Gal4 UAS-*Shi^{ts}*) caused no preference for alcohol at 50/100 E/A dose compared to controls (Figure 8.1G). These results show that DA in PAM neurons are required for the formation of EDAP since silencing these neurons prevented flies from showing alcohol preference in the Café assay after prior ethanol exposure. TH-Gal4 DA neurons are on the other hand required for naïve alcohol aversion since silencing these neurons caused naïve preference in flies.

We next investigated whether DA release in these distinct subsets of DA neurons is the main cause of NAA and EDAP in flies. Using the Gal4-UAS system, we tested whether loss of DA expression via RNAi-mediated knockdown of tyrosine hydroxylase (UAS-*TH-RNAi*) phenocopied silencing TH- and PAM-Gal4 neurons with UAS-*Shi^{ts}*. This indeed was the case, as loss of DA expression (UAS-*TH-RNAi*) in PAM-Gal4 neurons prevented the formation of EDAP at 50/100 E/A, while loss in TH-Gal4 neurons caused NAA at 0/150 E/A (Figure 8.2B, 8.3B). Taken together, we show and confirm that distinct DA neurons are required to influence both the appetitive and aversive properties of alcohol and that the PAM neurons is required for the formation of EDAP.

PAM neurons innervate the MBs to affect acquisition of EDAP

In figure 8.1G, it was observed that pretreatment of ethanol in PAM-Gal4 UAS-*TrpA^{ts}* flies did change the level of alcohol preference at different doses (30/120, 50/100 E/A) when compared to their respective controls (Figure 8.1G, 8.2B). These results suggest that DA neurons expressed by PAM-Gal4 might be required during pretreatment to

affect alcohol preference. We addressed this hypothesis by investigating when are DA neurons expressed of PAM-Gal4 required to affect alcohol preference. Is it required during the pretreatment or during the choice (café) test? Therefore, we temporally controlled silencing or activating PAM-Gal4 neurons either during ethanol pretreatment or during the choice (café assay) experiment by switching the temperature to 30°C (Figure 8.2C). Silencing PAM-Gal4 neurons at 30°C only during pretreatment (Figure 8.2D) but not during the test (Figure 8.2E) prevented the formation of EDAP at 50/100 E/A dose. The mushroom bodies (MB), which are innervated by PAM DA neurons (Burke et al., 2012) were also required only during pretreatment to affect EDAP as silencing MB neurons using UAS-Shi^{ts} prevented the formation of EDAP at 50/100 E/A dose (Figure 8.2F, 8.2G). Taken together, our data shows that PAM neurons innervation of the MB is required for EDAP acquisition in flies.

TH-Gal4 neurons are required during the test for NAA

Unlike the PAM DA neurons, pretreatment with ethanol at different doses did not lead to formation of EDAP in TH-Gal4 UAS-Shi^{ts} flies suggesting that DA neurons expressed by TH-Gal4 are required only during the test to affect alcohol preference (Figure 8.1F, 8.3C). Indeed this is the case as activating (using UAS-$TrpA^{ts}$) TH-Gal4 neurons at 30°C (Figure 8.3B) during the test but not during pretreatment affected alcohol preference at different doses (0/150 and 50/100 E/A, Figure 8.3D, 8.3E). Therefore, our results show that distinct DA neurons have different timing and valence with PAM-Gal4 neurons required during pretreatment for rewarding/reinforcing (EDAP)

valence while TH-Gal4 DA neurons are required during the test for aversive (NAA) valence.

We next investigated whether alcohol influences DA release in TH-Gal4 DA neurons by expressing Chrimson (UAS-*Chrimson*), a red light-drivable channelrhodopsin (Klapoetke et al., 2014) in TH-Gal4 flies. Using fast-scan cyclic voltammetry (Vickrey et al., 2009), we measured red light stimulated DA release in dissected adult TH-Gal4 fly brains before and after addition of either vehicle or different concentrations of ethanol. Red light stimulation of alcohol treated brains caused an increase in DA release when compared to vehicle-treated brains with significant changes apparent at a 5mM ethanol concentration (Figure 8.3F-G). Taken together, our results suggest that acute exposure of alcohol potentiates DA release in TH-Gal4 DA neurons leading to NAA in flies.

DA Neurons expressed in the PPL1, PPM1/2 and PPM3 are required for NAA

To specifically identify what DA neurons expressed by TH-Gal4 are important for NAA, we obtained five transgenic Gal4 driver lines containing different regions of the TH genomic locus (TH-C'-, D'-, D4-, F2-, and C1-Gal4), and had limited or no expression in some DA neurons cluster required for NAA expressed by TH-Gal4 (Liu et al., 2012). Since DA neurons expressed by TH-Gal4 flies are required only during the café test for NAA (Figure 8.3D, 8.3E), we investigated whether silencing these five Gal4 lines during the café test at 30°C affected alcohol preference at 0/150 E/A dose. Three (TH-D1, -C1, -D'-Gal4) of the five Gal4 lines tested (UAS-*Shi*^{ts}) are required for NAA since silencing them caused naïve alcohol preference compared to their respective controls (UAS-*GFP*,

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Figure 8.5). The remaining 2 lines (TH-C'- and –F2- Gal4) did not affect NAA, suggesting that DA neurons expressed by these lines are not needed for NAA (Supplemental Figure 8.1). By assessing and eliminating DA neurons that are and are not required for NAA based on their location (Liu et al., 2012), our data suggest that DA neurons required for NAA are distributed within the PPL1, PPM1/2 and PPM3 clusters (Data not shown).

One PPL1 neuron's innervation of the fan-shaped body (FSB) is sufficient for NAA.

Since PPL1 neurons were expressed in all three Gal4 lines required for NAA (data not shown), we obtained PPL1-specific DA neuron Gal4 lines and focused on identifying what PPL1 DA neurons are sufficient for NAA. Three of the four Gal4 lines tested (065B, 504B and 502B), did not affect NAA, since silencing (UAS-Shi^{ts}) these PPL1-specific DA neurons at 30°C in flies showed similar aversion to alcohol as their respective controls (UAS-GFP, Figure 8.3H). However, silencing one PPL1-specific DA Gal4 (439B) line caused NAA. Although all PPL1-Gal4 lines tested have projections to the MBs, 439B was the only line to show NAA (Figure 8.3H). This suggests that PPL1 neurons expressed by 439B-Gal4 projects somewhere different compared to the other Gal4 lines (065B, 504B and 502B) tested. This is indeed the case, since immuno-staining of the 439B-Gal4 fly' brain using a UAS-GFP reporter showed that one PPL1 neuron (green, Figure 8.3J) projects to the FSB (Figure 8.3I-J). Furthermore, we show that activation of FSB neurons (23E10-Gal4) using UAS-$TrpA^{ts}$ causes naïve preference in flies while

silencing FSB neurons causes naïve aversion similar to controls (UAS-*GFP,* Figure

8.3H). This suggest that 439B-PPL1 DA neuron has an inhibitory effect on the function

of the FSB, as FSB activation promotes naïve preference not aversion. These results

taken together suggest that although most PPL1 neurons innervate the MB (Liu et al.,

2012; Mao and Davis, 2009), they are not required for NAA, while PPL1 neuron's

innervation of the FSB is sufficient for NAA.

DA neural projections to FSB and MB is necessary and sufficient for NAA and

EDAP in the fly brain respectively

To prove that the FSB is required for NAA, we carried out RNAi-mediated

knockdown of DA D1 and D2 receptors (UAS-*Dop1R1-RNAi*, UAS-*Dop1R2-RNAi*)

specifically in the FSB and MB. Pan-neuronal knockdown of D2 DA receptor (elav-Gal4

UAS-*Dop2R1-RNAi*) did not perturb alcohol preference when compared to controls

(Figure 8.4A, 8.4E). However, pan-neuronal knockdown of D1 DA receptor (elav-Gal4

UAS-*Dop1R1-RNAi*) in flies to show naïve preference at 0/150 E/A when compared to

controls (Figure 8.4A, 8.4D). Therefore, we carried out MB- and FSB-Gal4 specific

experiments using UAS-*Dop1R1-RNAi*. We show that knockdown of DA D1 receptor in

the FSB (23E10-Gal4 UAS-*Dop1R1-RNAi*) led to naïve preference at 0/150 E/A when

compared to controls (Figure 8.4G). Unlike the FSB, knockdown of D1 receptor in the

MB showed similar aversion to ethanol as controls at 0/150 E/A but prevented the

formation of EDAP at 50/100 E/A compared to controls (Figure 8.4F).

Similar to pan-neuronal knockdown of D1 receptor, flies carrying a mutation in the

D1 DA receptor (D1R^{f026}) show naïve preference at 0/150 E/A compared to controls, and can be rescued using mifepristone induced elav-GeneSwith-Gal4 to drive UAS-expression of D1R (UAS-D1R) within D1R^{f026} mutant (elav-Gal4; D1R^{f026}, Figure 8.4H). However, UAS-D1R expression in the FSB or MB of D1R^{f026}, either rescued naïve preference or EDAP as FSB-Gal4 expression rescued naïve preference but not EDAP (Figure 8.4H-I), while MB-Gal4 expression failed to rescued EDAP but not naïve preference when compared to elav-Gal4; D1R^{f026} (Figure 8.4H-I). Therefore, we next investigated whether a combination of FSB- and MB-Gal4 driven UAS-D1R expression within D1R^{f026} was sufficient enough for normal ethanol preference in D1R^{f026} flies. This was the case indeed as FSB- and MB-Gal4 driven UAS-D1R expression in D1R^{f026} flies caused normal alcohol preference similar to elav-Gal4; D1R^{f026} flies (Figure 8.4H-I). Taken together, our results confirm that dopaminergic innervations of the FSB and MB are necessary and sufficient for normal alcohol preference with PAM neuronal projections to the MB required for EDAP while one PPL1 neuron's projection to the FSB is required for NAA in flies (Figure 8.4J).

Discussion

DA has been shown to influence several complex behaviors in flies, ranging from sleep to aggression (Alekseyenko et al., 2013; Ueno et al., 2012; Waddell, 2013; Waddell and Quinn, 2001) and our study in this café paradigm supports a model were distinct DA neuro-circuits (PPL1➔FSB, PAM➔MB) modulates opposing valences of alcohol in flies similar to mammals (Lammel et al., 2012). Our results also indicate that

unlike mammals, high levels of dopamine in the fly brain determines whether a fly will avoid alcohol even though distinct neural circuits control its opposing valence. Though this is opposite of the effects of DA in mammals with high levels predicting reward (Bromberg-Martin et al., 2010; Schultz, 2002), high dopamine levels might have evolved as an ecological mechanism for flies and other insects to avoid plant-based insecticides such as cocaine (which increases DA concentrations in synapses), that mammals do not innately possess (Nathanson et al., 1993; Sovik et al., 2014; Sullivan et al., 2008).

We show that DA neurons expressed by TH-Gal4 are required during the test but not during alcohol preexposure to regulate alcohol avoidance in flies. This suggests that DA neurons are activated during a fly's first exposure to ethanol. This appears to be the case as a low concentration of ethanol i.e. 5mM given acutely was able in stimulate DA release when compared to controls. We were unable to obtain any readings/measure of DA release from PAM-Gal4 neurons, but our results show that PAM neurons are required only during ethanol pretreatment for EDAP. Therefore, PAM neurons must also be activated by ethanol but the question that remains is whether [DA] release in PAM is similar or different from TH-Gal4 neurons since both subsets are required at different time scales to regulate distinct aspects of alcohol preference.

PAM neurons' innervation of the MB is required for acquisition. Our results also indicate that although memories are processed by the MBs, the MB is not required for retrieval of EDAP in flies. Although surprising, it is important to note that not all memories are stored in one common-purpose brain center i.e. the MB, as visual pattern memories for example are stored in the fly FSB (Liu et al., 2006). This result is further

supported by the fact that even though different lobes of the MBs are required for both the formation and retrieval of aversive and rewarding odor memories, and are innervated by PPL1 DA neurons (Sejourne et al., 2011; Vogt et al., 2014; Xie et al., 2013), the MB is not required for NAA (Figure 6.2G-F). Our results however suggest that one PPL1 neurons' innervation of the FSB is sufficient for NAA in flies. Therefore by studying different subsets of DA neurons, we were able to reinforce previous data showing that different anatomical structures are required for different aspects of alcohol preference (Ojelade et al., 2015).

Silencing PPL1 DA neuron has similar effect on naïve preference as activating FSB neurons unlike silencing the FSB. This suggests that PPL1 DA neurons inhibit activation of the FSB. Although, we focused on identifying what PPL1 DA neurons are sufficient for NAA, we also identified that some PPM3 neurons are required for NAA. PPL1 and PPM3 have both been shown to be required for ethanol-related behaviors, with PPM3 neurons being required for ethanol-induced locomotor activity and positive ethanol-oviposition place preference while PPL1 is required for negative ethanol-oviposition place preference (Azanchi et al., 2013; Kong et al., 2010). Although both PPL1 and PPM3 neurons project to the FSB, PPM3 also projects to the ellipsoid body (EB) of the fly brain and it is this projection that is believed to be required ethanol-induced locomotor activity (Kong et al., 2010). It is therefore possible that PPL1 and PPM3 projections to the FSB but not the EB are required for NAA in flies. Interestingly, projections to the FSB from the PPM3 and PPL1 cluster were recently identified to be required for wakefulness in flies (Liu et al., 2012; Ueno et al., 2012). This suggests that

the role of PPL1➔FSB neuro-circuit in alcohol aversion and arousal might not be mutually exclusive and further research will be required to evaluate this connection.

Our results show that the PAM and PPL1 neurons are sufficient at different time scales for alcohol preference. It is therefore possible that alcohol's effect on these DA neurons could also be time specific i.e. its effects on the release of DA in these distinct circuits (i.e. PPL1➔FSB and PAM➔MB) might determine its transition from an aversive to a rewarding valence. One possible way transition from NAA to EDAP could occur might depend on how the reinforcing stimuli of alcohol are represented by PAM DA neurons. Research shows that different subsets of PAM neurons mediate either short-term (STM) or long-term (LTM) reward memories and might have variable neuronal representations of LTM and STM inputs for different rewarding stimuli such as sugar and alcohol (Yamagata et al., 2015). Therefore, development of EDAP after a single pretreatment of alcohol would depend on how the appetitive properties of alcohol are represented by PAM DA inputs. Additionally, recent studies show that some MB output axons terminate on dendrites of PAM DA neurons that innervate Kenyon cells innervating the same MB output neurons (MBONs) thereby creating a feedback loop (Aso et al., 2014). This connection may provide a positive feedback loop, as most of these MBONs send glutamatergic inputs to PAM DA neurons, leading to increased DA release that promotes further development of EDAP. Since PPL1 and PAM innervation of the MB and FSB respectively is sufficient for normal ethanol preference, comparing dopamine release probabilities within these circuits after ethanol exposure might help decipher how the transition between opposing valences occurs.

One last possibility is that connectivity between the FSB and MB creates causal relationship affecting the transition from NAA and EDAP. In mammals for instance, circuit connections between the centromedial amygdala (CeM) and nucleus accumbens can causally affect the negative and positive emotional valences produced by each structure respectively. For inhibition of CeM projectors impairs fear conditioning and enhances reward conditioning and vice versa, while inhibition of the nucleus accumbens projectors impairs reward conditioning and enhances fear conditioning and vice versa (Namburi et al., 2015). Although 439B-PPL1 DA neuron projects to the FSB, it also has dendritic arborizations at the MB lobes (Aso et al., 2014; Mao and Davis, 2009). It is likely that some MBONs might send GABAergic inputs to 439B-PPL1 DA neuron causing decreased DA release and inhibition to the FSB, resulting in NAA inhibition and EDAP enhancement. Therefore studies investigating the connectivity between the central complex (containing the FSB and EB) and the MB would help address whether there is a causal transition between valences similar to mammals. Nevertheless, our results show that the role of DA in assigning valence to drugs of abuse is evolutionarily conserved between mammals and insects (Lammel et al., 2012; Waddell, 2013).

Acknowledgements

Experimental Methods

Fly husbandry and Genetics

Drosophila melanogaster were raised in a 12:12 hr. Light/Dark cycle on a standard cornmeal/molasses diet at 25°C with 70% humidity, except for temperature sensitive experiments, which used 30°C during the experiments. White Berlin served as the genetic background for all experiments (unless explicitly stated). Transgenic Gal4 driver lines containing different regions of the TH genomic locus (TH-C'-, D'-, D4-, F2-, and C1-Gal4) were obtained from the Dr. Wu. PPL1 specific-Gal4 lines were obtained from Dr. Karla Kaun. Other transgenic lines were obtained from the Bloomington stock center.

Drug feeding protocols

Pharmacological treatment with 3IY (Sigma) and L-Dopa (Sigma) were carried out as previously described (Bainton et al., 2000). 3-iodo tyrosine (3-IY) (10mg/ml) or L-Dopa (1mg/ml) was dissolved in solutions containing 250nM sucrose. Flies were pre-fed in a modified CAFÉ assay in rectangular 4-well plates (127.8 Å~ 85.5 mm, Thermo Scientific; Fig. 5). Food was provided in 0.2 ml tubes with a 27 G needle hole at the bottom for drinking access, a 27 G hole atop for pressure equilibration and a 25 G hole atop for filling with solution. Flies were fed 3IY for a period of 48-hours and L-Dopa for 24-hours in the modified Café apparatus. For the elav-GeneSwitch Gal4 experiments, food-deprived flies were fed with 0.5 mM mifepristone (RU486) for 3 hours prior to pretreatment to ethanol.

Booz-o-mat exposure

Exposure paradigm used is as previously described (Peru et al., 2014). The day before ethanol vapor exposure, male flies were collected in groups of 30 and put on un-yeasted food. The following day, flies were transferred into the Booz-o-mat apparatus for a 20 minute exposure at desired ethanol to air ratio (E/A) as described (Wolf et al., 2002). Temperature-sensitive experiments using UAS-Shi^{ts} and UAS-$TrpA^{ts}$ were allowed to acclimate at 30°C for 20 minutes in the before starting the 20 minutes exposure at 30°C. Flies were then transferred to vials and placed into a 25°C/70% humidity incubator for a 24-hour recovery period.

Capillary Feeder (Café) assay

24-hours after recovery from ethanol preexposure, 15 flies were placed into each well of the Café assay apparatus as described (Ojelade et al., 2015). Preference assay was carried out for 16-hours.

Immunohistochemistry

Immunohistochemistry was performed as previously described (Wu and Luo, 2006). To visualize 439B-PPL1-GAL4 expressions in the brain, a *pJFRC225-5xUAS-IVS-myr::smGFP-FLAG* (*smGFP-FLAG*) reporter probe (Viswanathan et al., 2015) was utilized. smGFP-FLAG (Green) was visualized with an anti-flag antibody. The presynaptic marker, mouse anti-nc82 antibody was used to label general neuropil/brain structure. The multicolor flip-out approach (MCFO, (Nern et al., 2015)) was used for stochastic labeling of 439B-PPL1 neurons.

Light-induced stimulation of DA neurons and Fast-scan Voltammetry

All chemicals were purchased from Sigma-Aldrich (St. Louis, MO) and all solutions were made with Milli-Q water (Millipore, Billerica, MA). Dissections, recordings, and calibrations were performed in a simple buffer solution (131.3 mM NaCl, 3.0 mM KCl, 10 mM NaH2PO4 monohydrate, 1.2 mM MgCl2 hexahydrate, 2.0 mM Na2SO4 anhydrous, 1.2 CaCl2 dihydrate, 11.1 mM glucose, 5.3 mM trehalose, pH = 7.4). Carbon fiber microelectrodes were fabricated from T-650 carbon fibers (a gift of Cytec Engineering Materials, West Patterson, NJ) and were used for fast-scan cyclic

voltammetry as previously described (Xiao et al., 2014*). Virgin females with UAS-CsChrimson (a chimera of CsChR and Chrimson) inserted in attp18 (Klapoetke et al., 2014) (a gift of Vivek Jayaraman) were crossed with TH-GAL4 (a gift of Jay Hirsh). Resulting heterozygous larvae were screened from light and raised on standard cornmeal food mixed 250:1 with 100 mM all-trans-retinal. A small amount of moistened Red Star yeast (Red Star, Milwaukie, WI) was placed on top of the food to promote egg laying.

For the protocerebrum recordings, brains were isolated into dissection buffer from adult flies using forceps under a dissection microscope, and the electrode was implanted from the lateral edge of the tissue into the dorsal medial protocerebrum. The electrode equilibrated in the tissue for 10 minutes prior to data collection and a baseline recording was taken for 10 seconds prior to stimulation. Red light estimated at 0.75 mW from a 617 nm fiber-coupled high-power LED with a 200 μm core optical cable (ThorLabs, Newton, NJ) was used to stimulate the CsChrimson ion channel. The TarHeel CV software (a gift of Mark Wightman) was used to control the light stimulation and to record the current from the applied voltage. After taking a baseline 2 second stimulation, 5 mM ethanol (10% in buffer) was added to the solution of fly buffer and then another stimulation was recorded after 5 minutes. The concentration of ethanol was increased to 15 mM and then to 45 mM. Stimulations were performed at each concentration five minutes after the ethanol was added to allow for equilibration. Adding increasing amounts of dissection buffer instead of ethanol was performed as a vehicle

control. Data are presented at mean +/- standard error of the mean (SEM) and graph error bars are SEM.

Statistics

Statistical significance of results in this manuscript was established using analyses of variance (ANOVAs) tests with GraphPad Prism software for Mac. For the post-hoc analyses, Dunnett's Test was applied to control for the multiple comparison when several groups were compared to the same control. Error bars in all experiments represent SEM. Significance was only attributed to experimental lines that were statistically different from their respective controls. Significance in all graphs shown are defined as *$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$.

A

tyrosine — TH → L-dopa — DDC → dopamine

48h 3IY feeding | 24h L-dopa feeding | 24h on food recovery | 16h CAFE assay

20 min **EtOH** pre-exposure

B pre-exposure: **0**/150

Alcohol Preference Index

fed: – | L-dopa | 3IY | 3IY + L-dopa

DA: less | – | more | –

C **50**/100

ns

fed: – | L-dopa | 3IY

DA: less | – | more

D

dopaminergic drivers:

PAM-Gal4

TH-Gal4

E

20 min **EtOH** pre-exposure

24h recovery | 16h CAFE assay

30°
25°

F *driver:* **TH-Gal4**

Alcohol Preference Index

Gal4; UAS -
TrpA^{ts}
ctl.
shi^{ts}

pre-exposure

0/150 | **30**/120 | **50**/100

EtOH/air

G **PAM-Gal4**

0/150 | **30**/120 | **50**/100

Figure 8.1: **Distinct dopaminergic neurons are required for NAA and EDAP.** (**A**) Experimental café paradigm. Flies fed 3-IY for 48-hrs and/or L-Dopa for 24-hrs were pre-exposed to different doses of ethanol for 20-minutes, 24-hrs before the Café. (**B**) Flies fed 3-IY show naïve preference to alcohol, while flies fed L-Dopa show increased aversion (NAA) compared to controls (*p < 0.05, n = 12-16). (**C**) Flies fed L-Dopa do not develop EDAP, while flies fed 3-IY show similar preference as controls (*p < 0.05, n = 12). (**D**) Fly brain showing the DA neurons expressed by PAM- and TH-Gal4. (**E**) Café paradigm using thermo-genetics. (**F**) Silencing TH-Gal4 neurons at 30°C with UAS-Shi^{ts} during pretreatment and the café test causes flies to exhibit naïve preference alcohol (*p < 0.05, n = 12) while activating with UAS-$TrpA^{ts}$ causes flies to be averse to alcohol in a dose dependent manner (**p < 0.01, n = 12). (**G**) Silencing PAM-Gal4 during pretreatment and the café prevent alcohol preference at a 50/100 dose (*p < 0.05, n = 12) while activating causes flies to like alcohol in a dose dependent manner (EDAP) (*p < 0.05 (0/150), **p < 0.01 (30/120), n = 12).

A

PAM-Gal4
MB-Gal4

B

PAM-Gal4

Alcohol Preference Index

UAS-
- *ctl.*
- *TH-RNAi*

**

0	50
150	100

C

20 min **EtOH** pre-exposure

24h recovery

16h CAFE assay

30° — D, F

25° — E, G

D

PAM-Gal4

Alcohol Preference Index

UAS -
- *TrpA^{ts}*
- *ctl.*
- *shi^{ts}*

pre-exposure

**

0	30	50
150	120	100

EtOH
air

E

MB-Gal4 >

*

0	50
150	100

F

PAM-Gal4

Alcohol Preference Index

UAS -
- *TrpA*
- *ctl.*
- *shi^{ts}*

pre-exposure

0	50
150	100

EtOH
air

G

MB-Gal4 >

0	50
150	100

208

Figure 8.2: **PAM DA neurons innervate the Mushroom bodies to affect EDAP acquisition**.
(**A**) Fly brain schematic highlighting PAM-Gal4 DA neurons. (**B**) RNAi-mediated knockdown of TH (UAS-*TH-RNAi*) in PAM-Gal4 DA neurons prevented alcohol preference in flies at 50/100 E/A compared to controls (**$p < 0.01$, $n = 12$). (**C**) Schematic for experimental café paradigm using thermo-genetics. (**D**) Silencing PAM-Gal4 DA neurons at 30°C with UAS-*Shi^{ts}* during pretreatment but not the test (**E**) causes flies not to prefer alcohol at 50/100 E/A (**$p < 0.01$, $n = 12$) while activating causes flies to like alcohol similar to controls. (**F**) Like PAM DA neurons, silencing MB-Gal4 neurons at 30°C with UAS-*Shi^{ts}* during pretreatment but not the test (**G**) also prevented alcohol preference at 50/100 E/A in flies when compared to controls (*$p < 0.05$, $n = 12$).

A **TH-Gal4**

B

Alcohol Preference Index

*

TH-Gal4 >
UAS -
▽ *TH-RNAi*
△ *TrpA*
◯ *GFP*
▽ *shi^{ts}*

pre-exposure

0	50
150	100

C

20 min **EtOH** pre-exposure

24h recovery   16h CAFE assay

30° — D
25° — E

D

EtOH
air

0	50
150	100

E

* **

0	50
150	100

F

45 mM ethanol
15
5
0

0.45 nA

2 s

G

ethanol
ctl.

normalized DA

Concentration (mM Ethanol)

H

Naive Alcohol Preference

Dopamine-   **FSB**- *Gal4 >*

D'   439B   504B   23E10   *UAS-*

■ *TrpA*
▦ *GFP*
□ *shi^{ts}*

**   *   **

I

439B > GFP

J
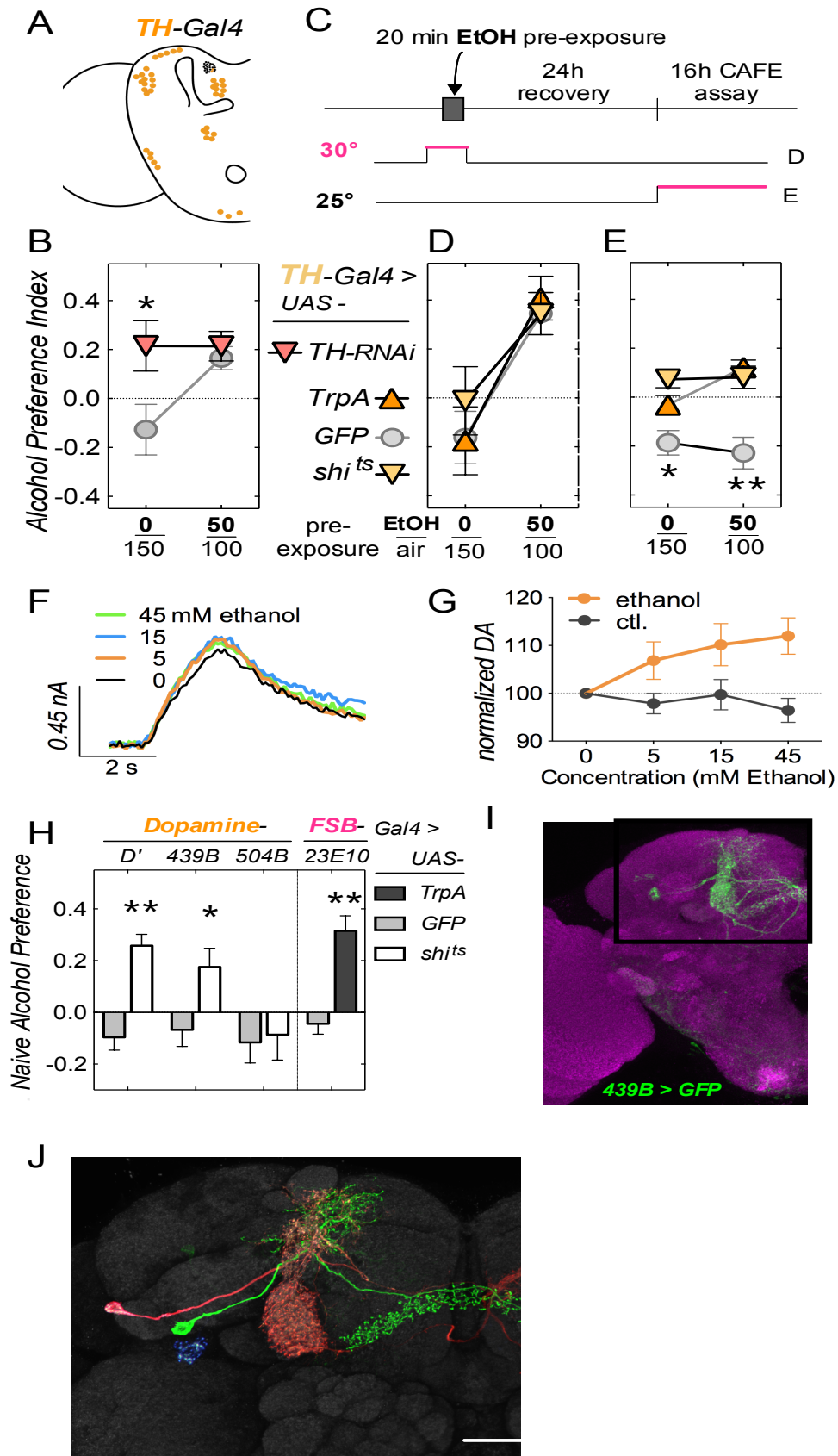
Figure 8.3: DA neurons expressed by TH-Gal4 are required during the café test for NAA.

(**A**) Fly brain schematic of TH-Gal4 DA neurons. (**B**) Expressing UAS-*TH-RNAi* in TH-Gal4 flies caused naïve alcohol preference (*p < 0.05, n = 12) (**C**) Cafe paradigm using thermo-genetics. (**D, E**) Silencing TH-Gal4 neurons with UAS-*Shi^{ts}* during (**D**) the test but not (**E**) during pretreatment caused increased aversion to alcohol in a dose-dependent manner (*p < 0.05 (0/150) n = 12-16, **p < 0.01 (50/100), n = 12). (**F**) Red-light stimulation versus time profiles from individual dissected adult TH-Gal4 brains before and after addition of either vehicle different [ethanol] (***p <0.001). (**G**) Graph comparing percent change from baseline of DA release in vehicle- vs. [ethanol]-treated TH-Gal4 brains after red light stimulation. (**H**) Silencing PPL1 DA neurons expressed by 439B-Gal4 caused naïve alcohol preference (*p < 0.05, n = 12). Silencing FSB (23E10-Gal4) neurons with UAS-*Shi^{ts}* during the test caused NAA while activating with UAS-TrpA^{ts} caused naïve preference at 0/150 E/A in flies (***p < 0.001, n = 12). (**I, J**) Immuno-staining of the fly brain showing that a PPL1 DA neuron (green, **J**) expressed by 439B-Gal4 innervates the FSB (**H**)

A *elav*-Gal4   B *MB*-Gal4   C *FSB*-Gal4

D *elav*-Gal4;
UAS - RNAi
⬇ -D1R
○ ctl.
⬇ -D2R

Alcohol Preference Index

**

E

pre-exposure   EtOH / air   0 / 150   50 / 100

F -Gal4;
UAS-
D1R-RNAi
FSB- ⬇
○ ctl.
MB- ⬇

*

G **

H -Gal4
D1R^UAS
☐ elavGS–
☐ elavGS+
MB- ☐
FSB- ☐
MB- &
FSB- ◑

*

I

J

PPL1-DAn   PAM-DAn   naïve avoidance   FSB   MB   experience-dependent preference
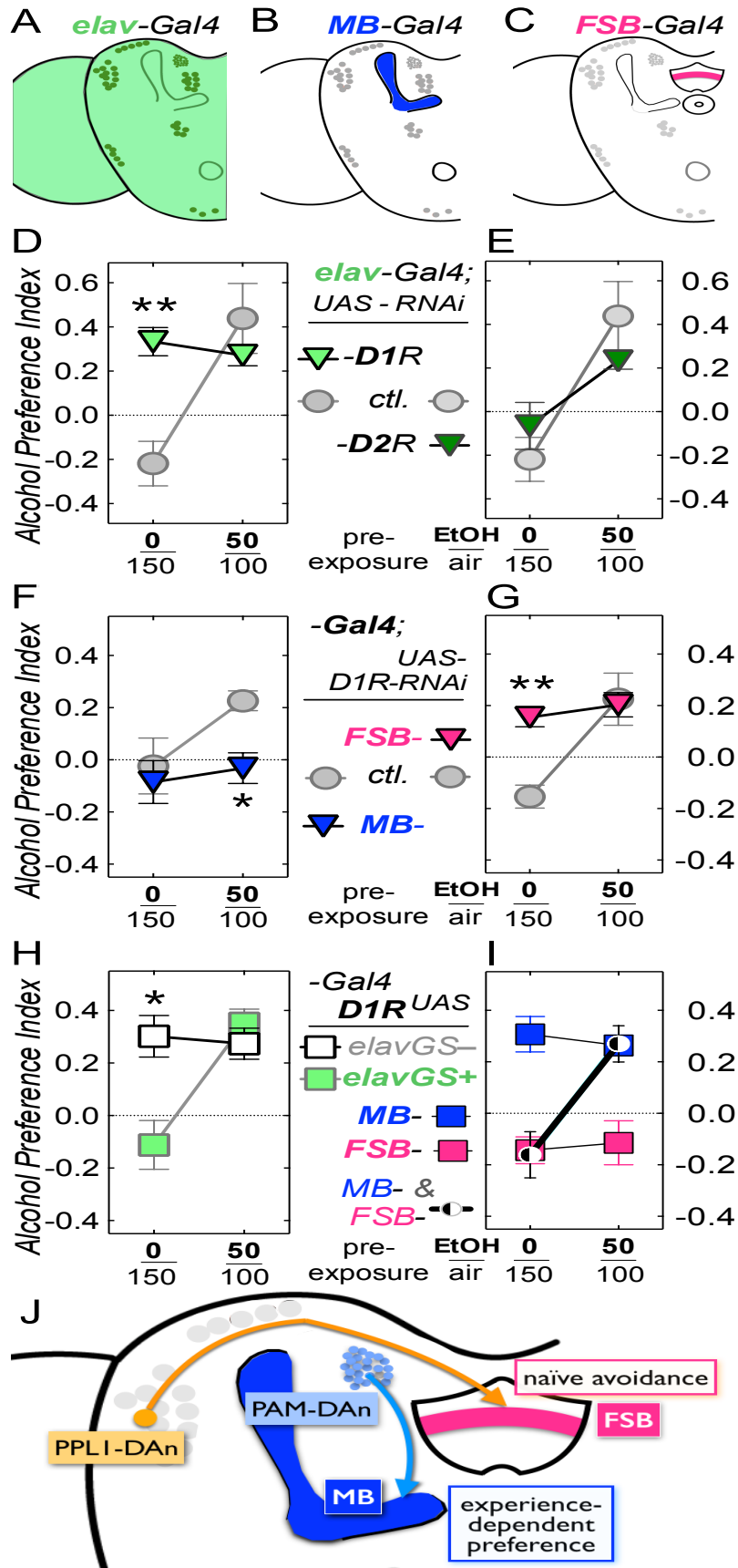
212

Figure 8.4. D1 receptor expression is necessary and sufficient in the FSB and MB for normal alcohol preference in flies.

Fly brain schematic showing (**A**) elav- (**B**) MB- and (**C**) FSB-Gal4 expression. (**D**) RNAi-knockdown of D1 receptor (D1R) in the CNS (elav-Gal4) causes naïve preference compared to controls (***p < 0.001, n $= 12$ (0/150)) unlike (**E**) D2Rs. (**F**) D1R knockdown in the MB prevents EDAP at 50/100 E/A (*p < 0.05, n $= 12$) while in the FSB (**G**) causes naïve preference at 0/150 E/A compared to controls (***p < 0.001, n $= 12$). (**H**) elav-Gal4 expression of D1R in the D1R^{f026} mutant background rescues alcohol preference compared to D1R^{f026} mutant (*p < 0.05, n$= 12$ (0/150)). (**I**) MB-Gal4 expression of D1R in D1R^{f026} mutant rescues EDAP at 50/100 E/A dose but not NAA at 0/150 E/A (**p < 0.01, n=12) while in the FSB rescues NAA but not EDAP at 50/100 E/A compared to elav-Gal4 rescue (*p < 0.05, n=12). Combination of MB-Gal4 and FSB-Gal4 expression of D1R in D1R^{f026} rescues alcohol preference like elav-Gal4. (**J**) Model showing that PAM and PPL1 neurons innervating the MB and FSB affect NAA and EDAP respectively in the fly brain.
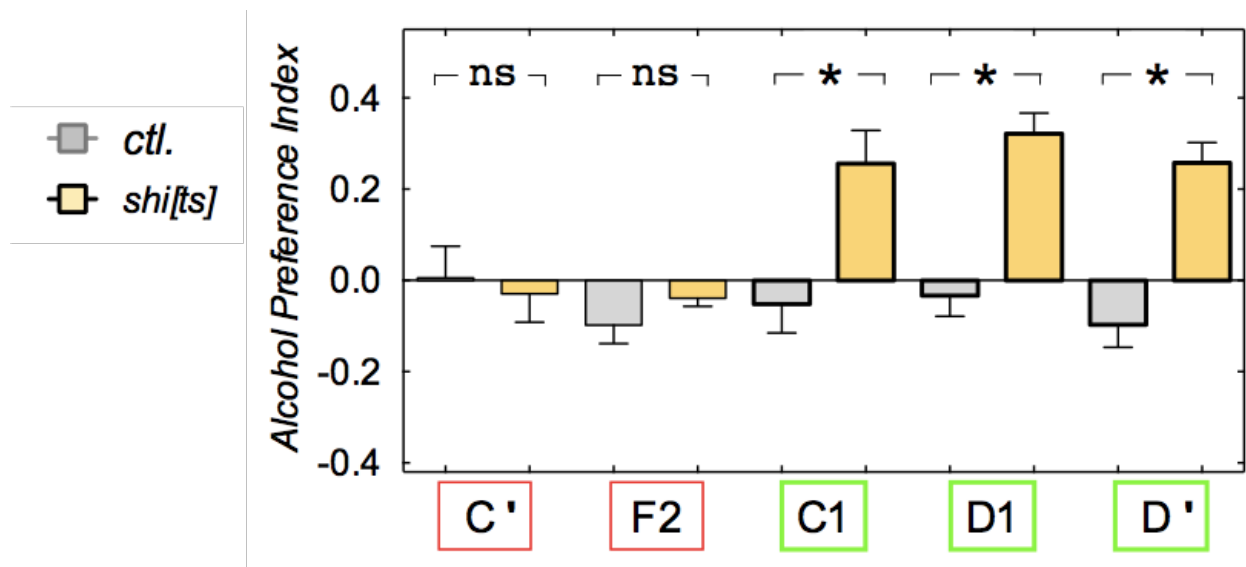
Figure 8.5: Identifying DA neurons required for NAA. 3 of 5 transgenic Gal4 driver lines containing different regions of the TH genomic locus (TH- D'-, D1-, and C1-Gal4) and expressing in different DA cluster caused naïve preference to alcohol compared to their respective controls (*$p < 0.05$, n = 12).

CHAPTER 9: Role of the actin cytoskeleton in naïve avoidance and experience-dependent alcohol preference

Introduction

The compulsion to drink irrespective of social norms and individual responsibilities is one of the hallmarks of alcoholism afflicting millions of people worldwide. Researchers have estimated that more than half of the risk for alcoholism can be attributed to an individual's hereditary predisposition (Enoch and Goldman, 1999). Therefore, identification and characterization of genes, signaling pathways, and neural circuits regulating alcohol-induced behaviors will help to produce better treatments for individuals with alcohol dependence.

The vinegar fly *Drosophila melanogaster* is a genetic tractable organism that has been utilized to study alcohol drinking. A two-bottle choice assay called CAFÉ, for capillary feeder, is a paradigm developed to measure a fly's preference for consuming alcohol (Ja et al., 2007). Here, flies can choose to drink from two capillaries containing liquid food (sucrose/yeast extract) with or without 15% ethanol. After the first day, flies show a slight preference for the capillary containing food and ethanol, but over the course of 3–5 days flies develop a clear preference of 2:1 for the food with ethanol, over the food without (Devineni and Heberlein, 2009). Alcohol also has aversive and appetitive properties in flies. For instance, in a modified capillary feeder (Café) assay called the FRAPPE assay, when given a choice between food and food-containing ethanol, naïve or mock exposed flies are averse to alcohol. However, if flies are allowed to experience alcohol 24 hours prior to a choice test, they show a dose dependent

preference for alcohol (Experience dependent alcohol preference (EDAP) (Peru y Colon de Portugal et al., 2014). Also, the experience of alcohol before the choice assay leads to lasting alcohol preference, since flies' preference for alcohol remained for over a period of 7 days (Peru YCDPL et al., 2014). Flies will also overcome/tolerate punishment (electric shocks, quinine) in order to consume alcohol suggesting that the intoxicating/pharmacodynamic effect of alcohol in flies is reinforcing/rewarding (Devineni and Heberlein, 2009; Kaun et al., 2012). Flies therefore exhibit naïve alcohol aversion (NAA), experience-dependent alcohol preference (EDAP), and other aspects of addiction like mammalian models (Pautassi et al., 2008; Pautassi et al., 2009)

In addition to behavioral responses to ethanol, genes affecting alcohol drinking are also similarly conserved in both flies and mammals. In particular, genes regulating the actin cytoskeleton have been implicated to play a role in alcohol-induced behaviors (Ojelade et al., 2013). The Rho family of small GTPases comprised of Rac1, Rho and Cdc42, regulates actin cytoskeletal dynamics and alcohol sensitivity in flies by cycling between an inactive GDP, and an active GTP forms (Rothenfluh et al., 2006). Furthermore, regulators of the small GTPase Rac1 such as Ras suppressor 1 (Rsu1) (Ojelade et al., 2015)) and the actin capping protein EPS8 (Disanza et al., 2004; Eddison et al., 2011; Offenhauser et al., 2006; Scita et al., 1999) also affect actin dynamics, ethanol sensitivity and alcohol consumption in both flies and mammals. Although actin is implicated to affect alcohol-induced behaviors in flies and humans, its role in alcohol drinking is still not clearly understood. Using a modified CAFE assay paradigm in addition to pharmaco-genetic tools, we demonstrate that the actin

cytoskeleton and Rac1 signaling is critically important for NAA and EDAP in the adult fly brain.

Results

Altering F-actin stability affects alcohol preference in flies.

In a 16-hour café assay, we show that mock-exposed (0/150 ETOH/Air (E/A)) wild-type flies show no preference for alcohol (NAA) (Figure 9.1A-B) (Ja et al., 2007). However, we see preference (EDAP) wirh exposure to vaporized alcohol, 24 hours prior to the cafe (Figure 9.1A-B). Using this paradigm, we explored whether perturbations of the actin cytoskeleton in flies affected alcohol preference. To do this, we stimulated F-actin polymerization by feeding flies Jasplakinolide (JPK), a compound promoting F-actin nucleation (Holzinger, 2009). Flies fed with JPK for 3 hours and then exposed to 0/150 E/A for 20 minutes (Wolf et al., 2002) show no preference for alcohol similar to wild type when given a choice between food and ethanol-containing food 24 hours later in the Café assay (Figure 9.1A, 9.1C). However at a 30/120 E/A dose, flies fed JPK show increased preference for alcohol when compared to controls. Conversely, flies treated with Latrunculin A (Lat. A), a compound that prevents F-actin polymerization show no preference for alcohol like wild type at 0/150 E/A but fail to develop EDAP at a 50/100 pretreatment dose (Figure 9.1A, 9.1D). We validated an increase or decrease of F-actin expression by JPK and Lat. A respectively by carrying out western blot analyses of F/G actin ratios in fly head lysates. Flies fed with Lat. A showed more G- than F-actin while flies fed with JPK showed more F- than G-actin (Figure 9.1E), quantified in figure 9.1F. Our results suggest that F-actin polymerization potentiates alcohol preference in

flies.

F-actin stability after ethanol pretreatment does not affect EDAP.

We next investigated whether timing of F-actin polymerization is important to affect alcohol preference in flies. To accomplish this, flies were fed either JPK or Lat. A 30-minutes after recovery from ethanol pretreatment for 3-hours (Figure 9.2A). 21 hours after drug feeding, flies fed JPK showed no preference to alcohol at a 0/150 E/A dose and showed no potentiation of EDAP at a 30/120 E/A dose when compared to controls and figure 9.1C (Figure 9.2B). Conversely, flies fed Lat. A after ethanol pretreatment showed similar effects on EDAP to flies fed before (Figure 9.1D, 9.2D). We next asked whether a 45-hour recovery after drug feeding (JPK and Lat. A, Figure 9.2A) would return alcohol preference back to wild type levels. This was not the case for Lat. A (Figure 9.2D). Although feeding JPK after ethanol pretreatment at both 21- and 45-hours showed trends toward preference at 0/150 E/A (Figure 9.2B-C), it was however not significant. These effects might however be due to the potency of the drugs used to alter actin polymerization in the adult fly brain. Nevertheless, our results indicate that timing of F-actin stability is important for alcohol preference with F-actin expression required during ethanol pretreatment to potentiate EDAP in flies (Figure 9.1C).

Rac1-mediated actin dynamics in the Mushroom Body regulates EDAP in flies.

The timing of F-actin stability is important for the potentiation of EDAP as shown in figure 9.1 and 9.2. This suggests that actin polymerization during alcohol

pretreatment is required to condition EDAP in flies. Since the Mushroom body (MB) is a brain center in the fly involved in associative olfactory learning and ethanol-reinforced odor preference (Burke et al., 2012; Kaun et al., 2011), we investigated whether altering actin dynamics in the MB affects EDAP acquisition. To alter actin dynamics specifically in the MB of the fly brain, we perturbed Rac1 signaling by expressing either the constitutively active (GTP-locked, Rac^{CA}) or dominant negative (GDP-locked, Rac^{DN}) forms of Rac1 in order to increase or decrease F-actin stability respectively. Using the GeneSwitch Gal4 system (Brand and Perrimon, 1993; Osterwalder et al., 2001), we induced UAS expression of either Rac^{DN} or Rac^{CA} in the MB by feeding flies mifepristone (RU486) 3 hours before alcohol pretreatment (Figure 9.3A-B). Expression of Rac^{CA} (UAS-Rac^{CA}) in the fly MB caused similar aversion as controls at 0/150 E/A, but prevented EDAP development at 50/100 E/A. Conversely, expression of Rac^{DN} (UAS- Rac^{DN}) in the fly mushroom bodies caused naïve preference at 0/150 E/A dose, and also increased flies' preference for alcohol at 50/100 E/A dose when compared to controls (UAS-GFP). These data were surprising as less F-actin gave more preference and NAA when compared to global effects of actin depolymerization. To validate our Rac1 expression results, we asked whether loss of Ras Suppressor 1 (Rsu1), an upstream inhibitor of Rac1 that affects ethanol sensitivity and preference in flies (Ojelade et al., 2015), showed similar effects like Rac^{CA} in the MB. Indeed, RNAi-mediated loss of Rsu1 using the inducible MB-GeneSwitch Gal4 system caused loss of EDAP at a 50/100 E/A dose (Figure 9.3C-D). These results suggest that altering actin dynamics by genetically manipulating Rac1 expression specifically in the MB has the

opposite effects on EDAP development when compared to global alteration of actin dynamics in the fly brain.

We next investigated when Rac1 expression in the MB is required for alcohol preference. To do this, we induced expression of either Rac^{CA} or Rac^{DN} by feeding flies RU486 after ethanol pretreatment (Figure 9.3E). Although expression of Rac^{DN} (UAS-Rac^{DN}) in the fly MB caused naïve preference at 0/150 E/A dose, it however showed similar preference to controls at a 50/100 E/A dose (Figure 9.3F-G). Additionally induction of Rac^{CA} expression or inhibition of Rsu1 expression in the MB after alcohol pretreatment did not affect development of EDAP at 50/100 when compared to controls and figure 9.3D (Figure 9.3F-G). Taken together, our results suggest that not only is the MB important for EDAP, proper Rac1 activity is required during ethanol pretreatment to affect EDAP acquisition in flies.

Cofilin affect EDAP in flies

To validate the relevance of Rac1 signaling for EDAP acquisition in the MB, we tested the effects of genetic perturbation of Rac1 downstream components. Cofilin, an actin-severing factor, is known to play a crucial role in mediating the cytoskeleton remodeling activity of Rac1. In this canonical pathway, Rac1 activity triggers sequential activation of PAK and LIMK, which in turn phosphorylates cofilin and inhibits its actin depolymerization activity (Meyer and Feldman, 2002; Ridley, 2006; Rogers et al., 2003; Shuai and Zhong, 2010). Activated Rac1 can therefore cause F-actin polymerization by its downstream inactivation/phosphorylation of cofilin. We therefore investigated

whether cofilin affected EDAP in the MB. To do this, we induced expression of either the constitutively active unphosphorylated form of cofilin (tsr^{CA}) or the dominant negative, pseudo-phosphorylated form of cofilin (tsr^{DN}) flies by feeding RU486 before or after ethanol pretreatment. Like expression of Rac^{CA} (Figure 9.3), expressing tsr^{DN} during ethanol pretreatment affected prevented flies from developing EDAP, but had no effect on EDAP after ethanol pretreatment when compared to controls (Figure 9.4A-D). On the other hand, induction of tsr^{CA} before or after ethanol pretreatment caused similar phenotypes as Rac^{DN} in the MB (Figure 9.3). Taken together, our results show that inactivating/phosphorylating cofilin EDAP in the MB (Figure 9.3-9.4).

Rac1 signaling in the Fan-shaped Body affects NAA in flies.

The fan-shaped body (FSB) of the fly brain has been implicated to play a role in ethanol-induced reward and addiction (see chapter 8). To investigate whether actin cytoskeletal rearrangement in the FSB have similar effects on alcohol preference like the MBs, we tested Rac1 signaling in the FSB. To prevent possible deleterious defects caused by perturbing Rac1 signaling in the FSB, we grew our fly strains at 18°C in order to dampen Gal4 activity and expression. We show that expression of Rac^{CA}, Rsu1 RNAi or tsr^{DN} in the FSB (by switching flies from 18°C to 29°C for 3 days, Figure 9.5A) causes naïve preference at a 0/150 E/A dose compared to controls while expression of Rac^{DN} or tsr^{CA} showed similar aversion to alcohol as wild type and prevented EDAP at a 50/100 E/A dose (Figure 9.5B-C). Furthermore, dampening expression of the UAS-transgenes by growing flies strains (Rac^{CA}, Rac^{DN}, Rsu1 RNAi, tsr^{DN}, and tsr^{CA}) at 18°C

had no effect on ethanol preference at 0/150 and 50/100 E/A dose (Figure 9.5D-E). To validate that 18°C caused reduced Gal4 expression in the FSB, western blot analyses were carried out with the UAS-GFP reporter line. Indeed, flies grown at 18°C show reduced expression of GFP compared to flies grown at 18°C and then switched to 29°C for 3 days (Figure 9.5F). These results show that Rac1-mediated changes in F-actin has the opposite effects in the FSB compared to the MB, and that the FSB might be required for promoting naïve alcohol avoidance since activating the Rac1 signaling pathway in the FSB affects NAA but not EDAP in flies.

Discussion

Chronic ethanol consumption leads to experience-dependent changes in brain function that manifest as physical dependence and addiction in humans. Using a modified café paradigm, we previously showed that the experience of alcohol 24-hours prior to a choice assay increases a fly's preference for alcohol that persist for a long period of time i.e. EDAP (Peru et al., 2014). Here, we provide evidence that EDAP acquisition in flies is contingent on proper growth and retraction of actin filaments with F-actin polymerization in the brain (i.e. JPK induced) leading to potentiation of EDAP, while depolymerization of F-actin (Lat. A induced) prevents EDAP development. Actin is highly enriched in dendritic spines, which are sites where > 90% of excitatory synapses are formed in the brain (Nimchinsky et al., 2002). Ethanol-induced neuronal activity at excitatory synapses is therefore required for actin-dependent changes in EDAP. Chronic ethanol exposure in hippocampal cell cultures leads to an increase in dendritic spine size, F-actin clusters, and requires enhanced synaptic expression of N-methyl D-

aspartate receptors (NMDAR) (Carpenter-Hyland et al., 2004), a key substrate for long term potentiation (LTP) in the brain (Ojelade et al., 2013). Although ethanol binds and inhibits NMDAR activity when given acutely (Popp and Dertien, 2008), studies suggest a cellular mechanism where neurons compensate for the continued inhibition of NMDAR through homeostatic adaptation that includes increases in F-actin stability and NMDAR abundance (Carpenter-Hyland and Chandler, 2006; Rothenfluh and Cowan, 2013). This appears to be the case as i) Inhibition NMDAR activity by ethanol when given acutely can be prevented by addition of phalloidin, an F-actin stabilizing agent (Popp and Dertien, 2008), and ii) Loss of the actin capping protein ESP8 in vivo causes chronic consumption of alcohol in a two-bottle choice assay in mice (Offenhauser et al., 2006) as a consequence of increased NMDA current and F-actin stability. Therefore, F-actin and NMDAR expression can aide in homeostatic adaptation to alcohol's inhibiting effects with NMDAR current leading to an increase in synapse size (dendritic spine) and F-actin expression during LTP induction. Concurrently, F-actin maintains or stabilizes the new morphology of the synapse, allowing for increased trafficking of NMDARs and other ion channels to the synapse (Carpenter-Hyland and Chandler, 2006) leading to EDAP acquisition.

Acquisition of EDAP requires precise timing of F-actin stability with alcohol pre-exposure, and is dependent on the MB, a brain site well characterized site for Pavlovian conditioning in flies (Margulies et al., 2005; Strausfeld et al., 1998; Tully and Quinn, 1985). A recent study observed that silencing MB neurons only during alcohol pretreatment but not during the 16-hrs choice test in our café paradigm affects

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acquisition of EDAP in flies (Ojelade et al., 2015, DA). Rac1 signaling in the MB is also required during but not after ethanol pretreatment to affect EDAP. Therefore, our results suggest that the MB acts as a coincidence detector that requires paired association of alcohol pretreatment with neuronal activity for EDAP acquisition. It also supports the idea that addictive drugs such as alcohol co-opt the learning-and-memory machinery to produce a long-lasting addictive state (i.e. EDAP) by utilizing existing plasticity mechanisms, including actin-mediated structural alterations.

Distinct anatomical regulation of Rac1-signaling via cofilin affects alcohol preference in flies.

F-actin lies downstream of Rac1 signaling with Rac1 activity adjusting the balance between F-actin polymerization and depolymerization. Rac1 activity (Rac^{CA}) enhances LTP induction by increasing F-actin stability and spine density while inhibition of Rac1 activity (Rac^{DN}) inhibits LTP induction (or enhance Long-term depression (LTD)) by decreasing F-actin stability and spine density (Impey et al., 2010; Schwechter and Tolias, 2013; Tashiro et al., 2000). Unlike global stability of F-actin in the brain potentiating EDAP acquisition, our results show that Rac^{CA} expression in the MB cause flies not to learn to acquire alcohol preference while Rac^{DN} potentiates learning in the MB. These results were surprising as research suggest that LTP maintenance and memory acquisition in the hippocampus (i.e. the site for learning and memory in mammals) is promoted through stability of F-actin (Lamprecht and LeDoux, 2004). It is possible that MB expressing Rac^{DN} flies might have an enhanced degree of learning

when compared to control flies which causes them to show naïve and acquire preference to alcohol (Ojelade et al., 2015 DA). Similar considerations exist for LTD induction (i.e. Rac^{DN} expression) in mammals, with some evidence suggesting a role for this form of plasticity in novelty processing and one-trial forms of spatial learning. For mutant mice lacking LIMK1, a protein functioning downstream of Rac1 and affecting F-actin stability, exhibit enhanced learning of context-dependent fear conditioning task, but showed defects at spatial memory task after repetitive training (Meng et al., 2002). Therefore, Rac^{DN} expression in brain structures required for associative learning and memory (such as the hippocampus and the MB) might heighten initial learning but is not required for acquisition of memory.

Different stages of memory (acquisition, consolidation, retrieval and forgetting) may require distinct cytoskeletal arrangements dependent on Rac1 signaling. For instance, a recent study in flies show that expression of Rac^{CA} in the MB promotes forgetting/memory decay while expression of Rac^{DN} in the MB inhibits forgetting in flies (Shuai et al., 2010). Rac^{DN} effects on MB-dependent EDAP is not due to forgetting since Rac^{CA} expression after alcohol pretreatment in the MB does not affect EDAP. It does however suggest that an appropriate level of Rac1 activity under basal conditions is important in the MB for maintaining normal learning and memory functions. This appears to be the case as enhanced Rac1 activity/LTP in the hippocampus does impair learning and memory performance in mice (Kim et al., 2009; Migaud et al., 1998; Oh et al., 2010) just as it impairs EDAP acquisition in the fly MB. Further experiments will be required to explore these possibilities in more depth.

Although Rac1's effect on alcohol preference appears bidirectional, our data suggest that Rac1 signaling in different structures of the fly brain affects distinct aspects of alcohol preference. Expression of Rac^{CA} in the FSB affects naïve preference but does not potentiate or prevent EDAP development in flies. This result is contrary to what is seen with Rac1 signaling in the MB. Rac1 signaling in the FSB does however mimic FSB's neuronal effects on alcohol preference (Ojelade et al., Submitted). This suggests that loss of Rac1-mediated F-actin stability silences neuronal activity in the FSB thereby promoting NAA in flies. Our results also support our previous study showing that loss of Rsu1 (an upstream inhibitor of Rac1) in the MB caused flies not to acquire ethanol preference i.e. EDAP, while outside the MBs loss of Rsu1 affected sedation-sensitivity and naïve consumption preference (Ojelade et al., 2015). Similarly, mouse studies have also shown differential effects of Rac1 signaling in the mammalian brain with suppression in the nucleus accumbens promoting conditioned place preference (CPP) for cocaine (Dietz et al., 2012), while global Rac1 activation (i.e. in a Kalirin7 knock-out mouse) led to reduced cocaine CPP (Kiraly et al., 2010).

Lastly, we show that Rac1 signaling via cofilin affects alcohol preference in flies with expression of either Rac^{CA} or tsr^{DN} preventing EDAP acquisition in the MB while allowing expression of either Rac^{CA} or tsr^{DN} in the FSB caused naïve preference in flies. Cofilin has previously been implicated in behavioral responses to other drugs of abuse such as cocaine in rodents. For example, cocaine CPP was enhanced by expression of Rac1^{DN} and by constitutively active cofilin, while Rac1^{CA} suppressed cocaine CPP (Dietz et al., 2012). Like flies, these proteins (Rac1, cofilin) are also required in mammals for

acquisition of drug-induced memories as experiments with photo-activatable protein showed that Rac1 is acutely required during the induction of place preference (Dietz et al., 2012). Our data therefore continues to extend previous findings by identifying that Rac1 signaling via cofilin in distinct brain circuits can differentially affect drug-induced behavior preference in flies and mammals (Ojelade et al., 2015).

Acknowledgements

Experimental methods

Fly husbandry and Genetics

Drosophila melanogaster were raised in a 12:12 hrs Light/Dark cycle on a standard cornmeal/molasses diet at 25°C with 70% humidity, except for temperature sensitive experiments, which used 18 or 29°C as indicated. w^{1118} served as the genetic background for all experiments (unless explicitly stated). All transgenic lines were obtained from the Bloomington stock center.

Drug feeding protocols

Pharmacological treatment with Latrunculin A (Lat. A) (Sigma-Aldrich, MO, USA) and Jasplakinolide (JPK) (Sigma-Aldrich, MO, USA) were carried out as follows. Flies were food deprived for a period of 16hrs and were then fed food containing 250nM sucrose and either 8ug/ml of Lat. A or 200nM JPK for 3hrs. After 30mins on food recovery, flies were then placed through the ethanol pre-exposure and café paradigm.

For the MB-GeneSwitch Gal4 experiments, food-deprived flies were fed with 0.5 mM mifepristone (RU486) for 3 hours prior to ethanol pre-exposure and café paradigm.

Booz-o-mat exposure

Exposure paradigm used is as previously described (Peru et al., 2014). The day before ethanol vapor exposure, male flies were collected in groups of 30 and put on un-yeasted food. The following day, flies were transferred into the Booz-o-mat apparatus for a 20 minute exposure at desired ethanol to air ratio (E/A) as described (Wolf et al., 2002).

Capillary Feeder (Café) assay

24-hours after recovery from, 15 flies were placed into each well of the Café assay apparatus as described (Ojelade et al., 2015). Preference assay was carried out for 16-hours.

G/F-actin In Vivo Assay

G/F-actin assay was performed according to the manufacturer's instructions (G/F-actin In Vivo Assay Kit, Cytoskeleton, Denver, CO). G- and F-actin bands on western blots were scanned by densitometry and the ratios of free G-actin to actin present as F-actin were calculated.

Statistics

Statistical significance of results in this manuscript was established using analyses of variance (ANOVAs) tests with GraphPad Prism software for Mac. For the post-hoc analyses, Dunnett's Test was applied to control for the multiple comparison when several groups were compared to the same control. Error bars in all experiments represent SEM. Significance was only attributed to experimental lines that were statistically different from their respective controls. Significance in all graphs show are defined as $*p < 0.05$, $**p < 0.01$, and $***p < 0.001$.

A

16h food deprivation | 3h drug feeding | 24h on food recovery | 16h CAFE assay

20 min **EtOH** pre-exposure

F/G actin determination

B

Alcohol Preference Index

| 0/150 | 20/130 | 40/110 | 60/90 | 80/70 |

C

▲ JPK
● *Ctl*

| 0/150 | 30/120 |

D

● *Ctl*
▽ Lat.A

| 0/150 | 50/100 | **EtOH**/air |

pre-exposure the day before

E

anti-actin blots

ctl.
JPK
Lat.A

G   F

ctl. JPK Lat.A

3% input

F
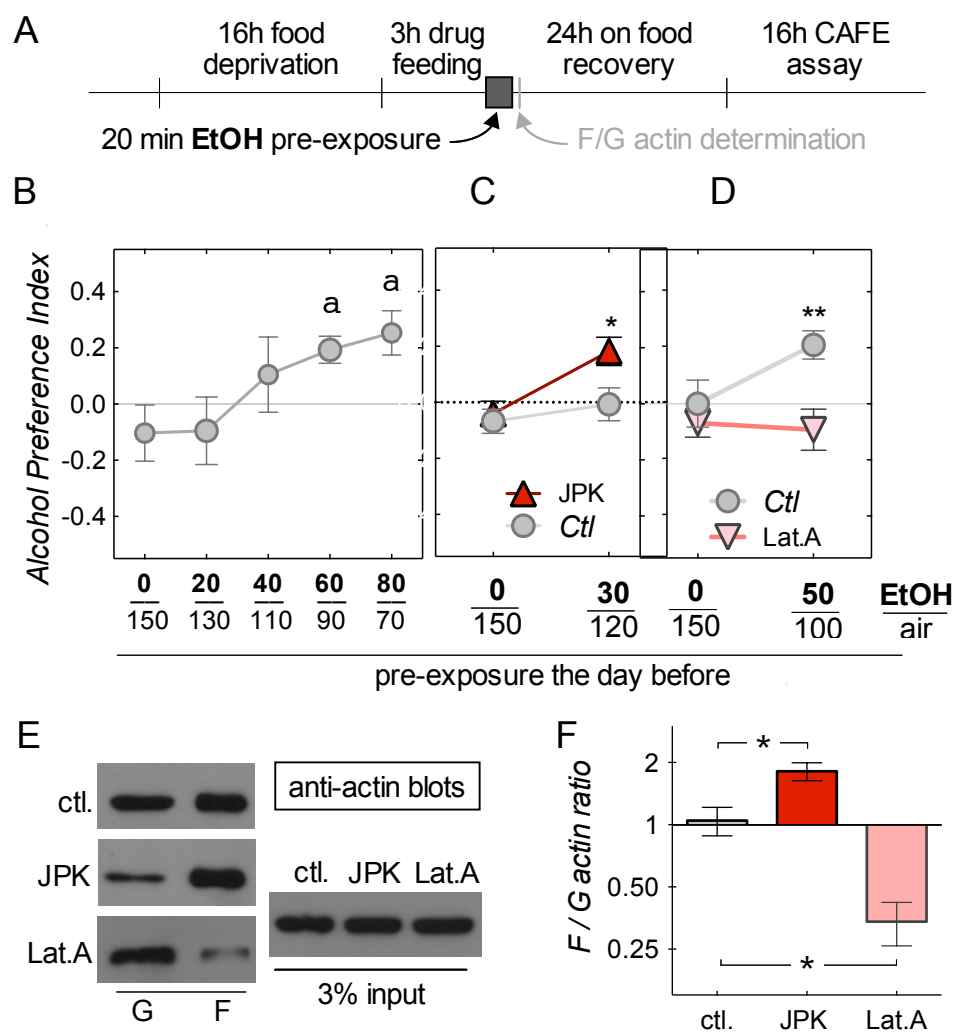
F / G actin ratio

ctl. JPK Lat.A

230

Figure 9.1: **Altering F-actin expression affects alcohol preference in flies.**
(**A**) Schematic for the experimental café paradigm. Flies were fed either Latrunculin A
(Lat. A) or Jasplakinolide (JPK) for 3-hrs and were then pre-exposed to different doses
of ethanol for 20-minutes, 24-hrs before the Café assay experiment. (**B**) Naïve flies
exhibit aversion to alcohol (NAA) in a 16-hour café assay (Ja et al., 2007), but show
EDAP in a dose-dependent manner if exposed to vaporized alcohol 24 hours prior to the
choice. (**C**) Flies with more F-actin (JPK fed flies) show similar aversion to alcohol like
controls at a 0/150 E/A dose, but show increased preference for alcohol when
compared to controls at 30/120 (*$p < 0.05$, n = 12-16). (**D**) Flies with less F-actin (Lat. A
fed flies) show similar aversion to alcohol like controls at a 0/150 E/A dose, but fail to
develop preference for alcohol when compared to controls at 50/100 (*$p < 0.05$, n = 12-
16). (**E**) Western blot images showing G- and F- actin protein expressions from brain
lysates of flies fed either JPK or Lat. A. (**F**) Quantification of F/G actin western blots (**E**)
showing increased F-actin in JPK fed flies and decreased F-actin in Lat. A fed flies (*$p <
0.05$, n = 3).

A
16h food deprivation   3h drug feeding   21 or 45h on food recovery   16h CAFE

20 min **EtOH** pre-exposure

30 min on food recovery

B   recovery: 21 hrs

C   45 hrs

Alcohol Preference Index

JPK
Ctl

EtOH / air pre-exposure

D   recovery: 21 hrs

E   45 hrs
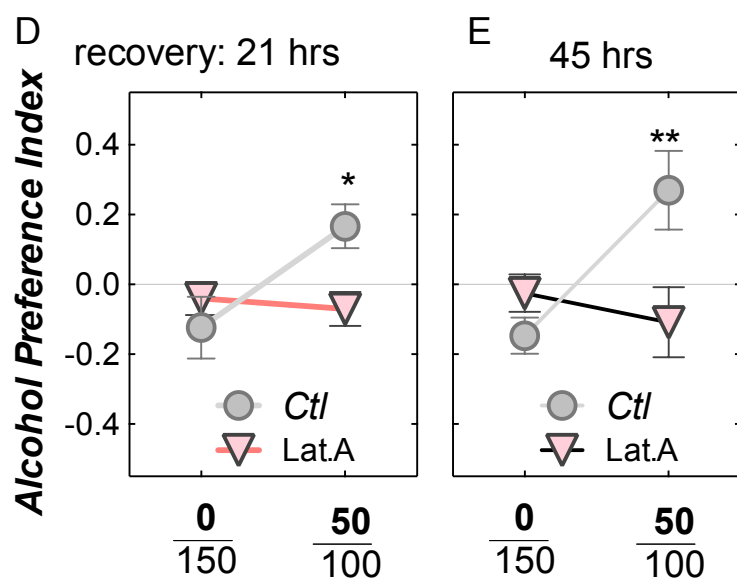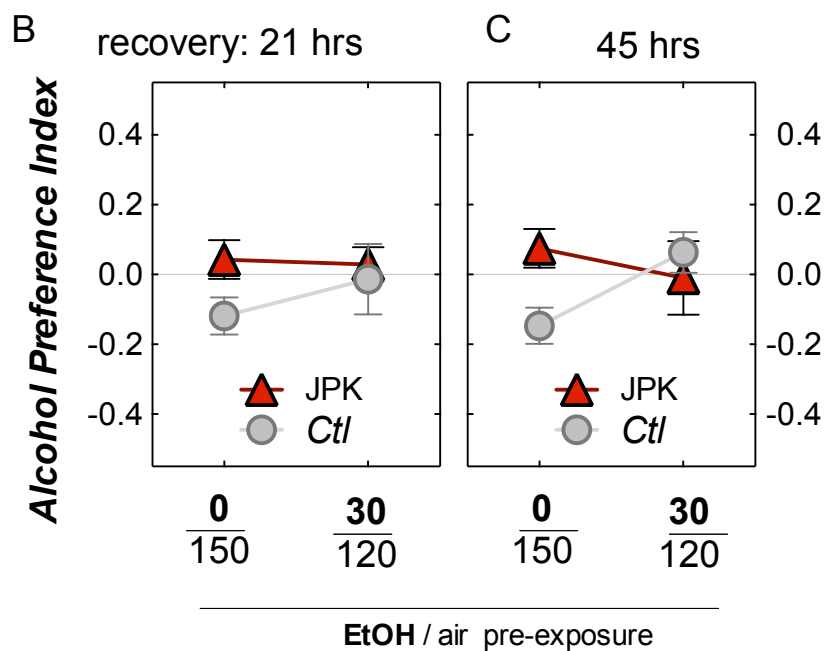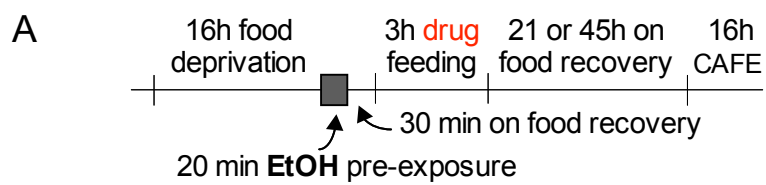
Alcohol Preference Index

Ctl
Lat.A

Figure 9.2: **F-actin stability after ethanol exposure does not affect alcohol preference in flies.**

(**A**) Schematic for the experimental café paradigm. Flies were fed either Latrunculin A (Lat. A) or Jasplakinolide (JPK) for 3-hrs after pre-exposed to different doses of ethanol for 20-minutes. Flies were then allowed to recover for 21 or 45-hrs before the Café assay experiment. (**B**) Flies fed JPK after pre-exposure show similar preference to alcohol as control at both a 0/150 and 50/100 E/A dose if allowed to recover for 21- or (**C**) 45-hrs. (**D**) Flies fed Lat. A after pre-exposure show similar preference to alcohol as control at 0/150 E/A dose, but fail to develop preference for alcohol when compared to controls at 50/100 if allowed to recover for 21- (*p < 0.05, n = 12-16) or (**E**) 45-hrs (**p < 0.01, n = 12-16).

A

B 20 min **EtOH** pre-exposure

3h ↓ 24h recovery 16h CAFE assay

RU 486:
+
−

expression:

C pre-exposure: **0** / 150

Alcohol Preference Index

**

UAS - *GFP* *Rsu1-RNAi* *CA Rac* *DN Rac*

D **50** / 100

*

**

**

GFP *Rsu1-RNAi* *CA Rac* *DN Rac*

MB-Geneswitch;

E 20 min **EtOH** pre-exposure

↓ 1h 3h 20h recovery 16h CAFE assay

RU 486:
+
−

expression:

F pre-exposure: **0** / 150

Alcohol Preference Index

**

UAS - *GFP* *Rsu1-RNAi* *CA Rac* *DN Rac*

G **50** / 100
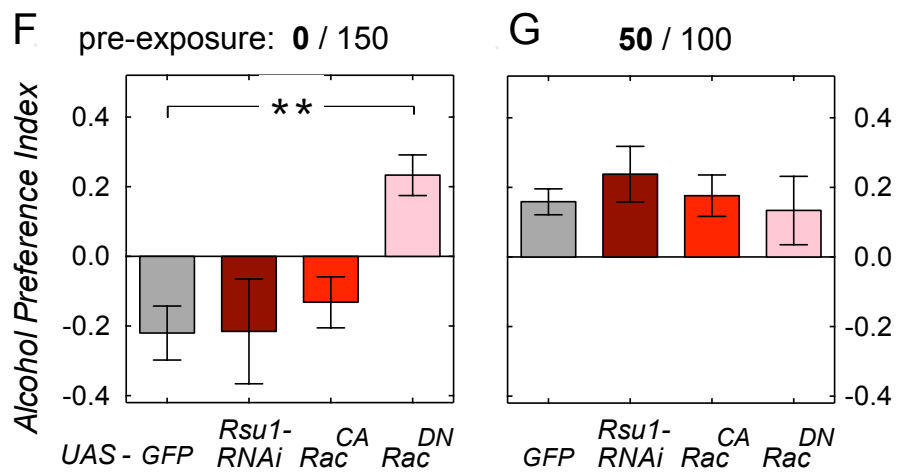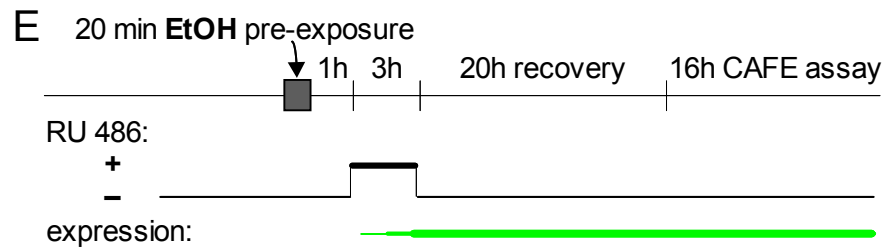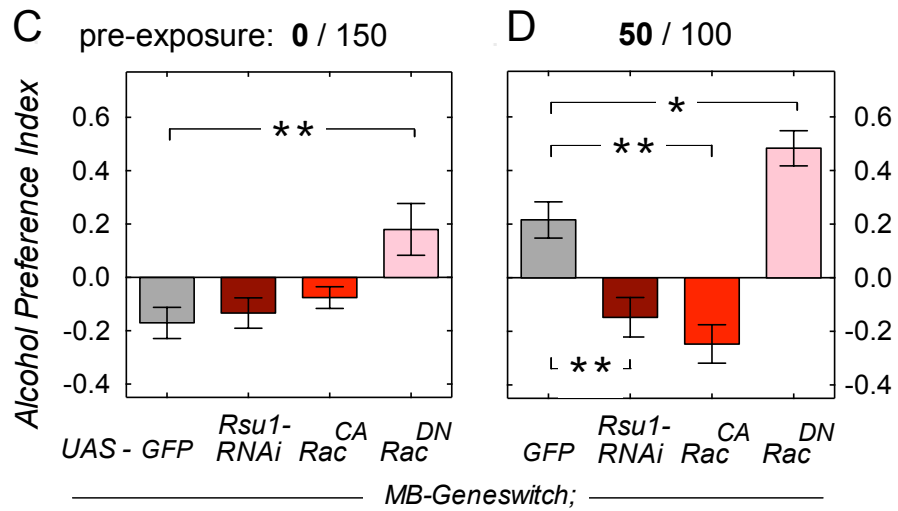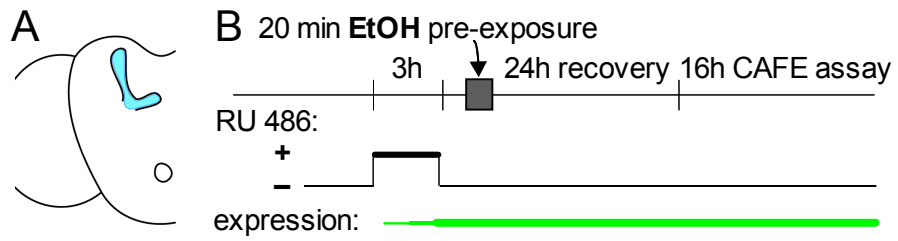
GFP *Rsu1-RNAi* *CA Rac* *DN Rac*

234

Figure 9.3: **Rac1 expression in the Mushroom Body is required for EDAP in flies.**
(**A**) Diagram showing the Mushroom body (MB) structure in the fly brain. (**B**) Schematic for the experimental café paradigm. Flies were fed RU486 for 3 hours and were then pre-exposed to different doses of ethanol for 20-minutes, 24-hrs before the Café assay experiment. (**C**) Flies fed RU486 to induce expression of the dominant negative form of Rac1 (Rac^{DN}) in the MB before pretreatment to ethanol show naïve preference at 0/150 E/A dose (**p < 0.01, n = 12-16) and (**D**) increase preference for alcohol at a 50/100 E/A dose compared to controls (UAS-GFP, *p < 0.05, n = 12-16), while flies fed RU486 to induced expression of the constitutively active form of Rac1 (Rac^{CA}) show no preference at 0/150 E/A dose and do not develop preference for alcohol at a 50/100 E/A dose compared to controls (**p < 0.01, n = 12-16). (**E**) Schematic for the experimental café paradigm. Flies were fed RU486 for 3 hours after pre-exposed to different doses of ethanol for 20-minutes, 24-hrs before the Café assay experiment. (F) Flies fed RU486 to induce expression of Rac^{DN} in the MB after pretreatment to ethanol show naïve preference at 0/150 E/A dose (**p < 0.01, n = 12-16) (**G**) but show similar preference for alcohol at a 50/100 E/A dose compared to controls, while flies fed RU486 to Rac^{CA} show no preference at 0/150 E/A dose and show similar preference for alcohol at a 50/100 E/A dose compared to controls.

A pre-exposure: **0** / 150

B **50** / 100

MB-Geneswitch;
(activation before ethanol pre-exposure)

C pre-exposure: **0** / 150

D **50** / 100

MB-Geneswitch;
(activation after ethanol pre-exposure)

Figure 9.4: **Rac1 functions through cofilin to affect EDAP in the fly MB.**
(**A**)) Flies fed RU486 to induce expression of the constitutive active form of cofilin (tsr^{CA}) in the MB before pretreatment to ethanol show naïve preference at 0/150 E/A dose (**$p < 0.01$, n = 12-16) and (**D**) increase preference for alcohol at a 50/100 E/A dose compared to controls (UAS-GFP, **$p < 0.01$, n = 12-16), while flies fed RU486 to induced expression of the dominant negative form of cofilin (tsr^{DN}) show no preference at 0/150 E/A dose and do not develop preference for alcohol at a 50/100 E/A dose compared to controls (**$p < 0.01$, n = 12-16). (**E**) Flies fed RU486 to induce expression of tsr^{CA} in the MB after pretreatment to ethanol show naïve preference at 0/150 E/A dose (**$p < 0.01$, n = 12-16) (**D**) but show similar preference for alcohol at a 50/100 E/A dose compared to controls, while flies fed RU486 to tsr^{DN} show no preference at 0/150 E/A dose and show similar preference for alcohol at a 50/100 E/A dose compared to controls.
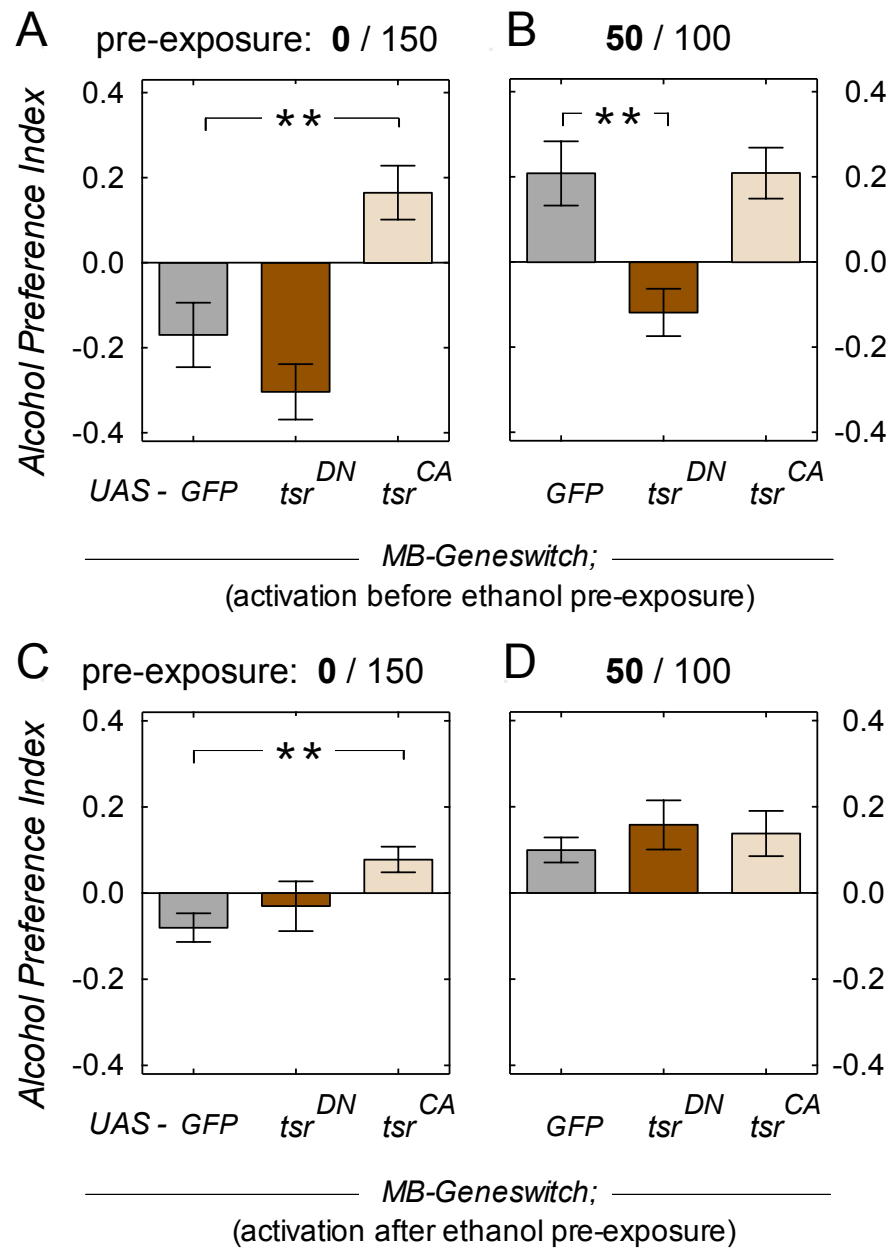
A   *FSB*-Gal4

B   20 min **EtOH** pre-exposure
    24h recovery   16h CAFE assay
    29°
    18°   3d   C, D   **25°**
    18°   E, F   **25°**

C   pre-exposure: **0** / 150
    Alcohol Preference Index
    GFP   Rac^CA   Rac^DN   Rsu1 RNAi   tsr^CA   tsr^DN

D   **50** / 100
    GFP   Rac^CA   Rac^DN   Rsu1 RNAi   tsr^CA   tsr^DN

E   pre-exposure: **0** / 150
    Alcohol Preference Index
    GFP   Rac^CA   Rac^DN   Rsu1 RNAi   tsr^CA   tsr^DN

F   **50** / 100
    GFP   Rac^CA   Rac^DN   Rsu1 RNAi   tsr^CA   tsr^DN
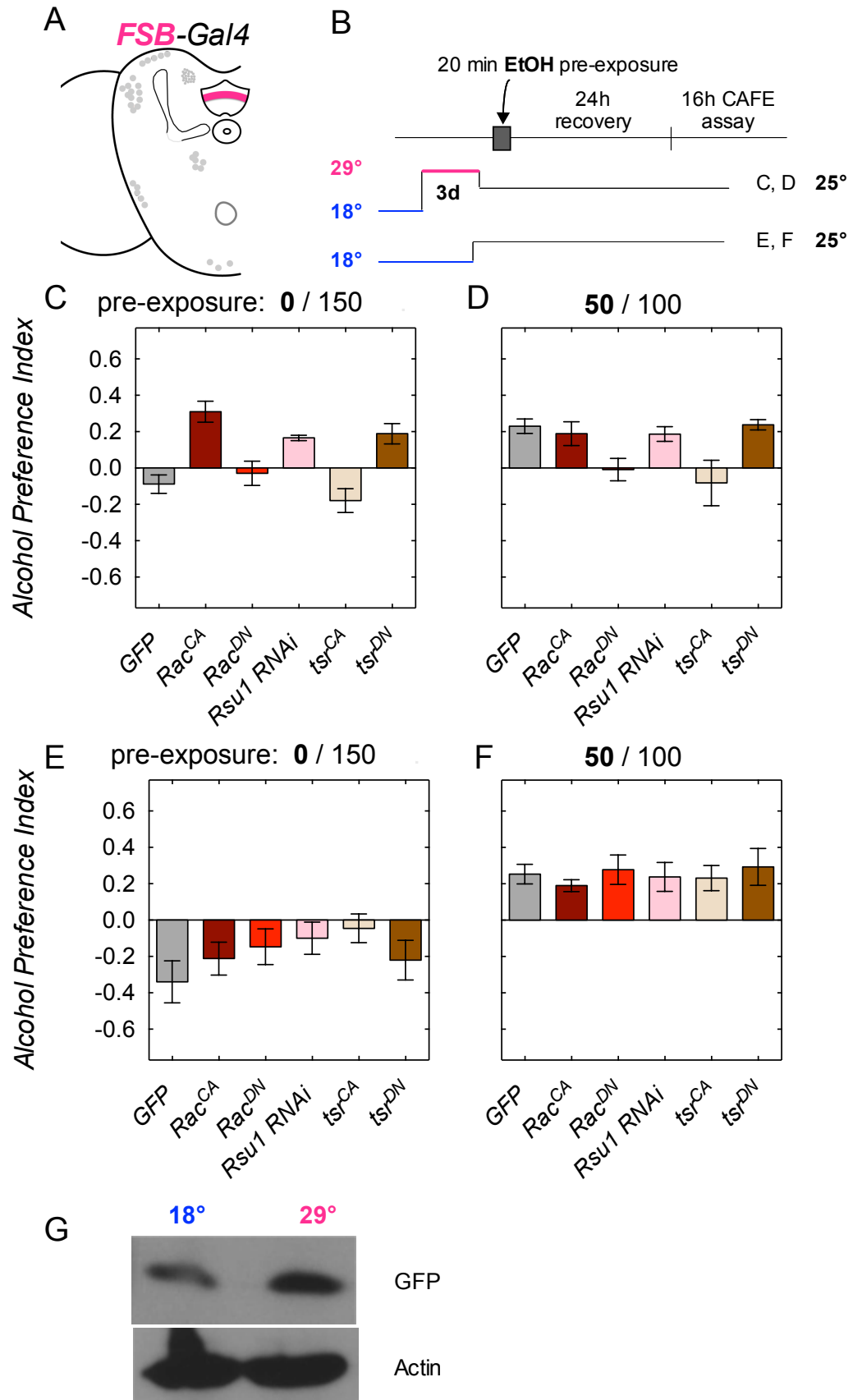
G   18°   29°
    GFP
    Actin

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Figure 9.5: **Rac1 signaling/F-actin stability is required in the Fan-shaped Body to affect for naïve alcohol aversion.**

(**A**) Schematic for the experimental café paradigm. (**B**) Flies were grown at 18°C (E,F) or placed after at 29°C for 3 days (C,D) and were then pre-exposed to different doses of ethanol for 20-minutes, 24-hrs before the Café assay experiment at room temperature. (**C-D**) Growing flies at 18°C and then allowing expression of Rac^{CA}, tsr^{DN}, and Rsu1 RNAi in the FSB by switching flies to 29°C for 3 days causes naïve preference in flies while expression of Rac^{DN} and tsr^{CA} cause similar aversion to alcohol as controls (GFP) at 0/150 E/A but affects EDAP acquisition at 50/100 E/A dose. (**D**) Schematic for the experimental café paradigm. Flies were grown at 18°C and after eclosion were then pre-exposed to different doses of ethanol for 20-minutes, 24-hrs before the Café assay experiment at room temperature. (**E-F**) Preventing Gal4 expression of Rac^{CA}, tsr^{DN}, tsr^{CA} and Rsu1 RNAi in the FSB by growing flies at 18°C but not switched to 29°C for 3 days did not affect (**E**) NAA or (**F**) EDAP in flies when compared to controls. (**G**) Western blot analysis shows reduced Gal4 expression of GFP in the FSB when grown at 18°C compared to flies switched to 29°C for 3 days.

CHAPTER 10: Summary and future directions

Rho family of small GTPases and acute ethanol responses

By carrying out a forward genetic screen, I identified Rsu1 as an actin regulator that functions specifically through the small GTPase Rac1 to regulate ethanol sensitivity in adult flies (See Chapter 4). In chapter 5, I provided evidence that different RhoGAP18B isoforms act on distinct subsets of Rho-family GTPases (i.e. Rho, Rac1, and Cdc42) to modulate cofilin activity, actin dynamics, and acute ethanol-induced behaviors in flies. RhoGAP18B-PC and -PD isoforms function through Rho and Rac1 to mediate the sedating effects of alcohol in flies (See Chapter 5; (Rothenfluh et al., 2006)). Meanwhile, RhoGAP18B-PA isoform functions through Cdc42 to give a distinctive effect on cell shape unlike Rho and Rac1 (or PC and PD). In addition, previous research suggests that PA recruits Cdc42 to regulate the stimulant effects of alcohol (i.e. ethanol-induced hyperactivity) but not ethanol sedation (Rothenfluh et al., 2006). Like Rho1 and Rac1, loss of Rsu1 leads to resistance to sedation and ethanol-induced hyperactivity (Figure 10.1, (Rothenfluh et al., 2006)). Since mutations in small GTPases and their regulators (Rsu1 and RhoGAP18B) do not cause any developmental anomalies or defects in the brain, it implies that alcohol recruits different small GTPases to induce certain levels of cofilin inactivity and actin rearrangements in the fly brain in order to elicit distinct acute ethanol behavior. That is, during hyperactivity, alcohol might recruit Cdc42 and not Rho and Rac1. However, alcohol inactivates Cdc42 over time and recruits Rho and Rac1 to affect ethanol sedation in flies (See Figure 10.2). To test this

hypothesis, cofilin phosphorylation, and the activity of small GTPases (Rho, Rac1, and Cdc42) should be tested in whole fly brains to see if they increase or decrease over time as a fly transition from alcohol-induced hyperactivity to –induced sedation. Secondly, live imaging studies in S2 cells should be carried out to see if changes in cell shape progresses from normal to stellate in the presence of alcohol as cofilin phosphorylation increases (Figure 10.2). Experimental evidences from S2 cell culture and flies supporting this hypothesis would give credence to continue testing our hypothesis in mammalian neuronal cells.
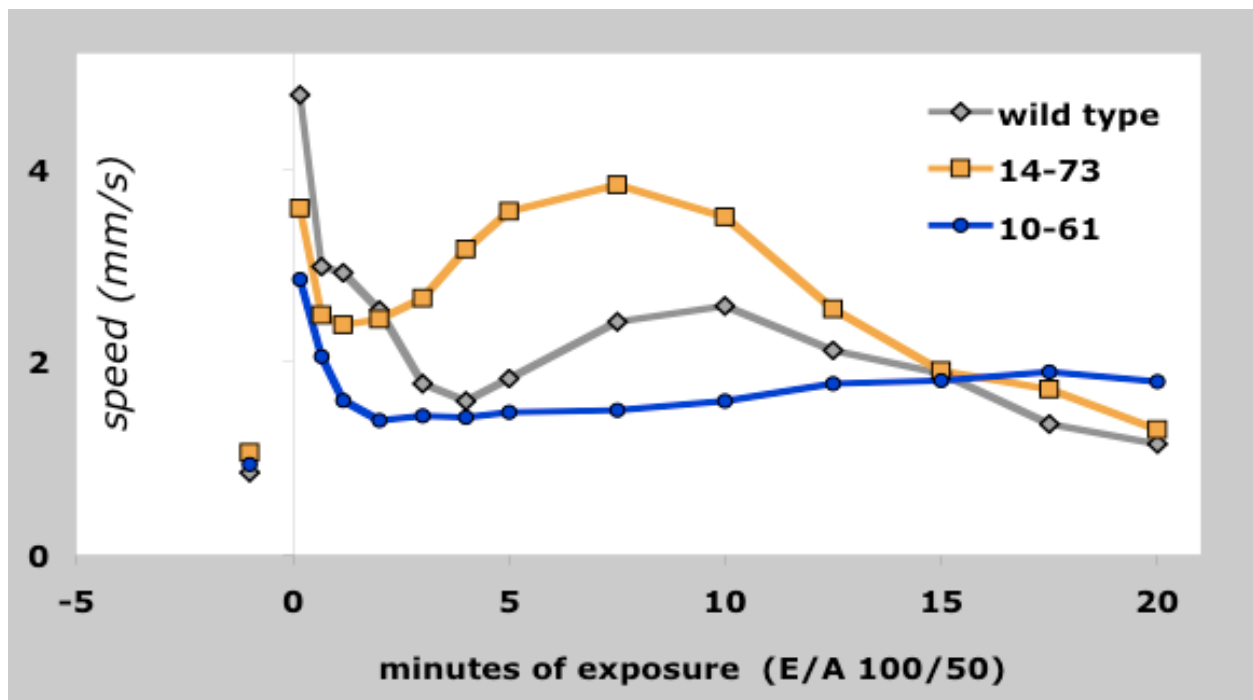

Figure 10.1. Loss of Rsu1 in flies affects acute ethanol responses. Locomotion tracking profiles of control (WT) and Rsu1 mutant (10-61) flies when exposed to a 100/50 EtOH/Air (E/A) dose. Loss of Rsu1 (which increases Rac1 activity) resulted in resistance to both ethanol-induced hyperactivity and –induced sedation (see chapter 4) when compared to controls.
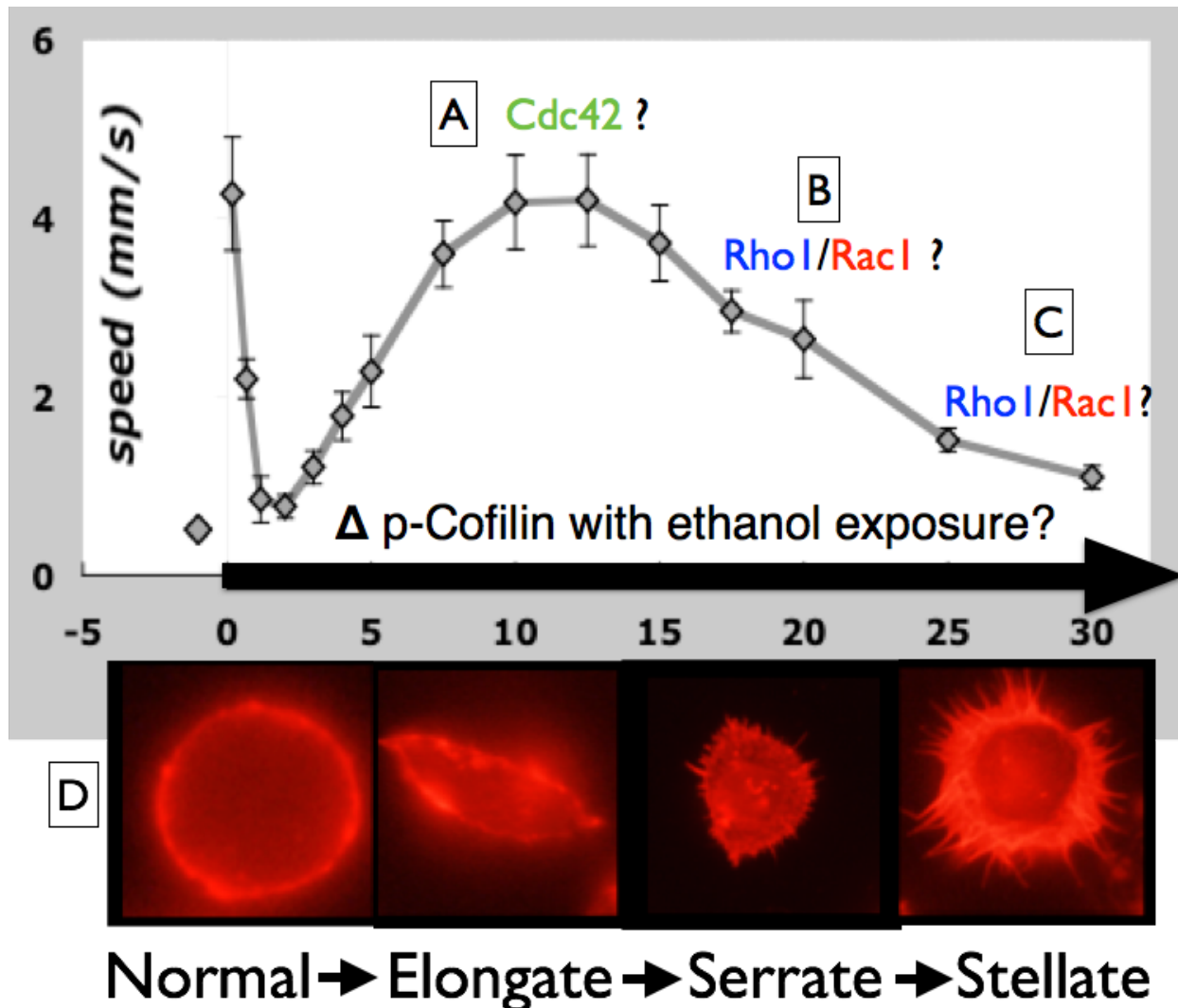
Figure 10.2. Hypothetical Model suggesting that distinct small GTPases are recruited to induce different levels of cofilin inactivity and actin-dynamics in order to affect acute alcohol-hyperactivity and –sedation. Hypothesis suggest that during ethanol exposure, small GTPases are recruit to induce certain levels of p-cofilin with (A) Cdc42 being recruited during the hyperactivity phase. As time goes on Cdc42 inactivated to prevent hyperactivity while induction of (B) Rac1 or Rho1 activity increases as flies begin to experience akinesia. (C) Rac1 or Rho1 activity continues to increase as fly begin to experience ethanol-induced sedation (i.e. Loss of righting reflex, see chapter 2). (D) Hypothesis also suggests that over time the levels of p-cofilin increases leading to actin dependent changes in cell shape (or synapse) thereby eliciting different acute alcohol behavior.

How does actin stabilize/maintain new configuration of the synapse?

Results in chapter 4 show that Rsu1 affects actin dynamics by functioning downstream of the cell adhesion molecule integrin. Integrins are bidirectional, allosteric signaling molecules capable of activating intracellular signaling pathways in response to changes in the extracellular environment (outside-in signaling) or altering cell adhesion as a consequence of intracellularly generated stimuli (inside-out signaling). Its possible that integrins are required for stabilizing actin-mediated structural plasticity (or LTP stabilization since mammalian studies i) show that loss of integrin signaling does not affect baseline synaptic transmission or induction of LTP (McGeachie et al., 2011) and ii) there is a critical window after LTP induction where loss of integrin signaling prevents LTP consolidation or stability through its interaction with the actin cytoskeleton (Bahr et al., 1997; Staubli et al., 1998). These observations indicate that integrin regulates LTP induction through the disassembly of actin filaments and synaptic adhesive contacts (including those mediated by integrins), which allows for the physical expansion of dendritic spines and the accommodation of new glutamate receptors (such as AMPAR, NMDAR) into the postsynaptic membrane. This new configuration is then stabilized through integrin-mediated re-assembly of F-actin networks and adhesive contacts to the ECM (See Figure 10.4 below). Integrins are highly expressed in the mushroom bodies of the fly brain. Therefore, future experiments should explore the potential role of integrin in alcohol preference.

What fly brain structures are required for alcohol-induced sedation, tolerance and EDAP retrieval?

My work with small GTPases and their regulators (i.e. Rsu1 and RhoGAP18B) have aided isolation of distinct circuits affecting ethanol-induced behavior ranging from acute to chronic alcohol consumption. My work (Ojelade et al., 2015) and others (Azanchi et al., 2013; Kaun et al., 2011; Kong et al., 2010; Rothenfluh et al., 2006) also suggest that ethanol-induced hyperactivity is regulated in the EB, NAA in the FSB, and EDAP acquisition in the MB of the fly brain. The next question to ask is what structures or circuits are required for ethanol-induced sedation and EDAP retrieval? Do the same circuit as NAA influence ethanol-induced sedation? And what structures/circuits are required for tolerance. To answer these questions, future studies can make use of Rsu1 mutant flies to delineate what structures are required for these behaviors. For instance, ics^{Gal4} expression rescues Rsu1-mutant's sedation phenotype in very limited parts of the fly brain, which includes the FSB, MB, and the Pars intercerebralis (PI) (See Figure 4.1). Preliminary data also shows that loss of Rsu1 prevents tolerance in flies when compared to controls and that it can be rescue with ics^{Gal4} expression pattern (Figure 10.3). Carrying out UAS-Rsu1 mutant rescue experiments with Gal4 lines specific for FSB, MB, EB, and the PI will provide evidence of what structures are required for sedation and tolerance.
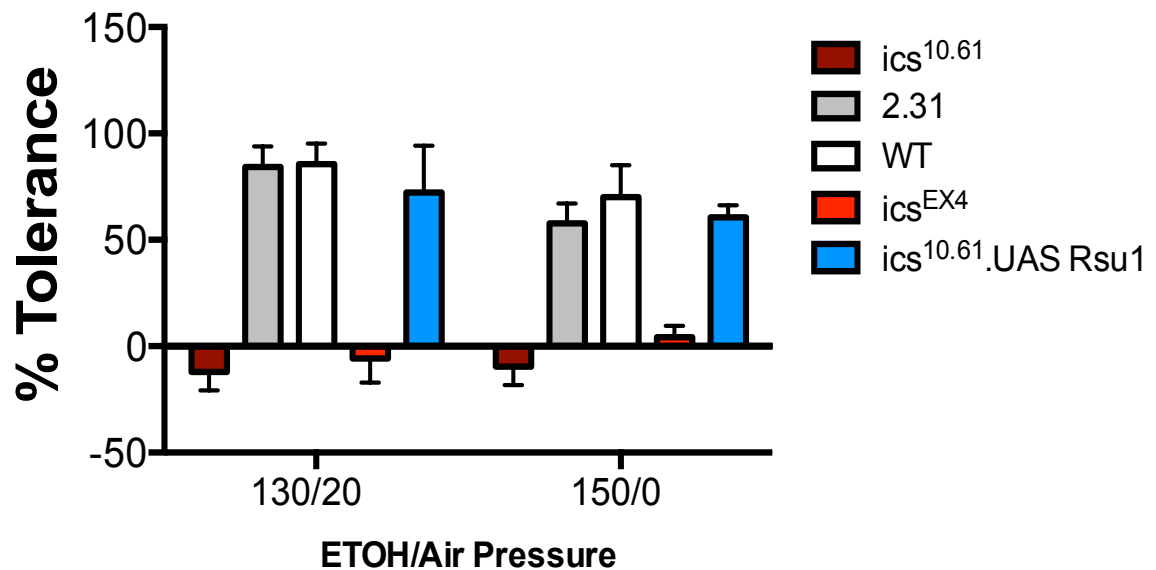
Figure 10.3. ***icarus,* encoding Rsu1, is required for tolerance in flies.** Using the Loss of righting reflex (LORR)**,** Graph shows %tolerance observed in flies 4 hours after the first exposure at different EtOH/Air (E/A) doses. *ics* mutant flies (*ics*[10.61], *ics*[EX4]) gain no tolerance to alcohol when compared to controls (WT, 2.31) at a 130/20 E/A and a 150/0 E/A dose. Expression of UAS-Rsu1 cDNA in the *ics* mutant background by using the Gal4 within *ics*[10.61] rescues tolerance at all does when compared to controls.

How does DA signal through Rac1 to affect alcohol preference in the FSB and MB respectively?

I provide evidence in chapter 8 that distinct subsets of DA neurons innervating the FSB and MB are necessary and sufficient to affect naïve alcohol aversion (NAA) and experience-dependent alcohol preference (EDAP) in flies. In chapter 9, expression of Rac1 and its regulator Rsu1 are required in the MB and FSB to affect NAA and EDAP. Results also show that Rac1-mediated changes in actin rearrangement in the FSB mirror dopaminergic-induced neuronal activity/silencing in these circuits. For example, Rac^{CA} expression in the FSB leads to naïve preference in flies similar to activation of FSB neurons with TrpA^{ts} suggesting that acute exposure of ethanol activates aversive PPL1 DA neuron resulting in FSB neuronal silencing thereby promoting NAA in flies (See Figure 8.3). As LTP induction/maintenance is dependent on actin-mediated changes in synapse morphology in different brain circuits (Lamprecht and LeDoux, 2004), I propose a model where dopamine functions upstream of Rac1-signaling to affect distinct aspects of alcohol preference in flies (See Figure 10.4 below).

A deeper understanding of how dopamine signals through Rac1 to affect different aspect of alcohol preference is first required to fully validate my proposed model. Results in chapter 8 implicate DopR1 (or D1R) important for NAA & EDAP in flies. DopR1 receptor expression in the FSB and MB was necessary and sufficient for normal alcohol preference in flies. My model suggests that DA neurons signal to DopR1 in the FB to inhibit the function of downstream neurons via Rac1 inactivation (See figure 10.4). Dopamine functions through D1- and D2- like receptors with D1 stimulating adenylate

cyclase activity and excitatory neurons potentiation while D2 inhibits adenylate cyclase activity and excitatory neurons potentiation (Cepeda et al., 1993; Hernandez-Echeagaray et al., 2004; Levine et al., 1996). Strausfeld and Hirth (2013) research suggests that the FSB predominantly contains GABAergic neurons similar to the striatum of the vertebrate brain. Additionally, studies in mammalian medium spiny striatal neurons suggest that GABAergic currents are principally reduced by D1 receptors (Hernández-Echeagaray et al., 2007). Since inactivation of Rac1 activity (Rac^{DN} expression) mimics silencing of the FSB, my data suggest a model where PPL1-DA induction in the FSB increases DopR1 activation thereby inhibiting postsynaptic GABAergic current/Rac1 signaling promoting NAA in flies. To test this hypothesis, I will first have to investigate whether loss of GABA receptors (GABARs) in the FSB affects NAA in flies. If it is true, loss of GABARs in the FSB should lead to NAA in flies. I would also investigate whether DopR1 is predominant in GABAergic neurons of the FSB similar to medium spiny neurons in the vertebrate striatum by carrying out immunohistochemistry assays.

Rac1 signaling has a different effect in the MB compared to the FSB. Data in chapter 8 & 9 show that EDAP acquisition in flies requires MB-dependent induction of neuronal activity during alcohol pretreatment (See Figure 8.2). This suggests that like mammals, alcohol co-opts the learning-and-memory machinery to produce a long-lasting addictive state in flies (Dietz et al., 2009). Activation of the MB promotes EDAP acquisition similar to neuronal activation of PAM-DA neuron (See chapter 8). These results indicate that PAM-DA signaling through the DopR1 must activate target neurons

in the MB to affect EDAP. Experience/activity dependent plasticity requires LTP induction/maintenance, and is dependent on an increase in F-actin amount (See chapter 9, (Lamprecht and LeDoux, 2004)). Surprisingly, Rac^{CA} expression increasing F-actin amount in the MB, prevented acquisition of EDAP in flies compared to controls. These results were unexpected (i.e. Rac^{CA} preventing EDAP acquisition) yet similar contradictions have been observed in mammals were enhanced Rac1- (Migaud et al., 1998), and NMDA-activity (LTP, (Kim et al., 2009)) affected hippocampal-dependent learning and memory. However, olfactory conditioning in flies has implicated that Rac1 activity decreases after 1 training session and continues to drop with repetitive training. These studies suggest that an appropriate level of Rac1 activity under basal conditions is important in the MB for maintaining normal learning and memory functions. Therefore continued LTP maintenance/F actin stability might make synapses in the MB less plastic (i.e. rigid, less motile) thereby preventing further acquisition of memory (Migaud et al., 1998). Since my studies and other researches used sledgehammer approaches (i.e. always on (Rac^{CA}) vs. off (Rac^{CA}) expression of Rac1)), a more appropriate experiment would be to express Rac^{CA} transiently in the MB during learning (i.e. ethanol pretreatment) and then turn it off after. This experiment would also help to answer the possibility of whether PAM-DA expression leads to transient Rac1-signaling in the MB during learning to promote EDAP. This might be the case as Rac1 appears to have a transient role during the induction of NMDA receptor-dependent LTP, but does not have an effect on LTP maintenance and expression (Martinez and Tejada-Simon, 2011).

PAM-DA neurons only innervate the β-lobes of the MB, which contains predominantly glutamatergic neurons (Aso et al., 2014; Mao and Davis, 2009). Rac1 activity in the β-lobes of the MB might thus have a different behavioral output compared to expression of Rac1 activity in all the overall MB structure since different lobes of the MB affect different valences and has different neurotransmitter inputs (i.e. GABA and acetylcholine). Therefore, Rac1 activity only in the MB β-lobes could lead to EDAP acquisition compared to its overall temporal summation in the MB. Studies observing structures and shapes of dendritic spines in the MB that is dependent on Rac^{CA} expression could yield some insights into how alcohol preference in learned in flies (Leiss et al., 2009). These future directions would help to understand how Rac1-mediated changes in actin function help sustain memory. It might also give insights into the vexing question of how alcohol can result in overlearning to the point of pathology if acute/chronic ethanol intoxication results in a depression in the capacity for learning and memory.
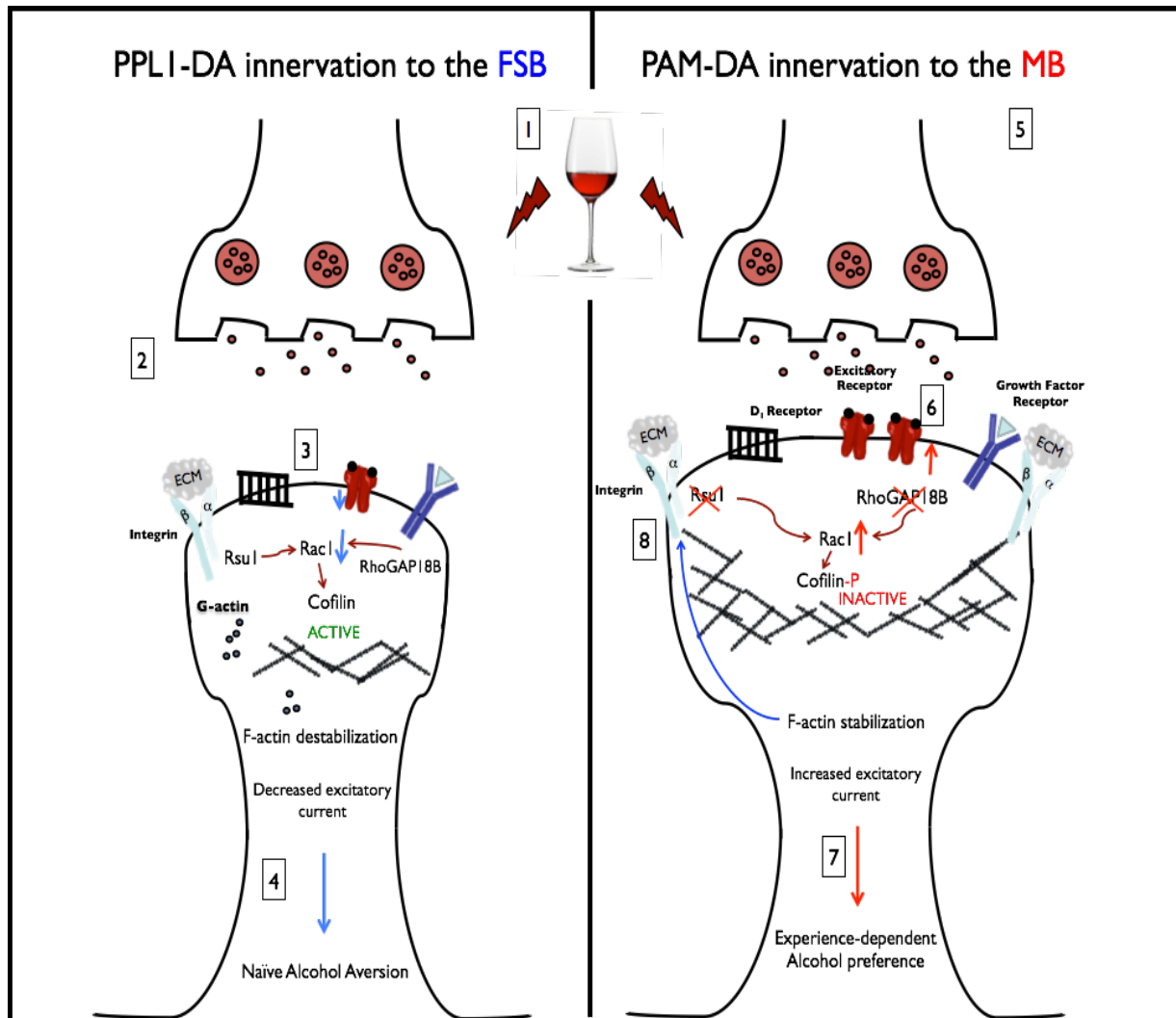
Figure 10.4. Proposed Model suggesting that Dopamine functions upstream of Rac1-signaling to affect NAA and EDAP in flies. (1) Acute exposure of ethanol to Naïve flies stimulates DA release from PPL1-DA neurons. (**2**) Dopamine from PPL1 neurons activates D1 receptor (D1R) on post-synaptic neurons in the FSB. (**3**) This leads to decreased current in FSB neurons either through D1R-dependent inactivation of NMDA receptors or D1R activation of GABA receptor. (**4**) Silencing of FSB neurons lead to downstream destabilization of actin rearrangement dependent on Rac1/cofilin signaling which affects the morphology of the synapse and promotes NAA in flies. (**5**) Ethanol also stimulates DA release from PAM-DA neurons (**6**) activating D1 receptor (D1R) on post-synaptic neurons in the MB. This leads to increased excitatory current through D1R activation of NMDA receptor resulting in downstream stabilization of F-actin dependent on Rac1/cofilin signaling increasing growth of the postsynapse. New morphology of the synapse (i.e. LTP maintenance) is maintained by integrin-dependent stability of F-actin thereby promoting EDAP.

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Naïve avoidance, Arousal and the FSB

Although, I focused on identifying what PPL1 DA neurons are sufficient for NAA, results from chapter 8 suggest that some PPM3 DA neurons are required for NAA. PPL1 and PPM3 are both required for ethanol-related behaviors, with PPM3 neurons important for ethanol-induced locomotor activity and positive ethanol-oviposition place preference (Azanchi et al., 2013; Kong et al., 2010). A future direction would be to identify what PPM3 neurons are required for NAA in our café paradigm. I hypothesize that PPM3➔FSB and not PPM3 ➔EB neurocircuit is required NAA since the EB has been implicated to regulate ethanol-induced hyperactivity. Also the FSB was sufficient to rescue Dopr1 mutant naïve preference in flies.

Why do naïve flies exhibit aversion to alcohol? One likely explanation is that flies do not like the taste of ethanol. Studies imply that mechano/chemosensory information from fly legs and proboscis (mouth) are processed in structures of the central complex (which includes the FSB). Therefore sensory modalities such as taste might alert a naïve fly to avoid approaching the alcohol solution by activating the PPL1➔FSB neurocircuit. Therefore, experiments investigating whether naïve flies show preference for differing [EtOH] or for bitter/aversive compounds like quinine and caffeine when PPL1 neurons are silenced would help to test this hypothesis.

The PPL1➔FSB circuit also control sleep/wake arousal in flies (Liu et al., 2012), implying that the FSB role in both alcohol aversion and arousal might not be mutually exclusive. If this is the case, do naïve flies show aversion to alcohol because it makes them hyper-aroused? Studies observing if sleep deprivation affects naïve alcohol

drinking or if transient acute ethanol exposure influences activity in the circadian monitor during both day and night phases in flies, would be a first step to address this questions. Since my data is consistent with the Rac1-signaling pathway functioning downstream of dopamine, investigating how Rac1/LIMK/Cofilin affects the arousal/NAA circuit is also warranted.

How does alcohol transition from NAA to EDAP in flies?

Preference for alcohol in flies spontaneously switch from a negative (NAA) to a positive (EDAP) valence after hours of conditioning in both our café paradigm and the conditioned odor-preference assay (Kaun et al., 2011). The kinetics of EDAP acquisition occurs slower than those observed in classical appetitive conditioning with EDAP taking 12-24 hour (Kaun et al., 2011; Peru et al., 2014) to acquire while sugar conditioning takes minutes to acquire after training (Burke &Waddell 2011; Fujita & Tanimura, 2011). Despite kinetic differences of behavioral changes, genes required for EDAP, which includes dopamine and actin regulators are inextricably required for appetitive conditioning of sugar. EDAP might thus depend on a transition from a reactive PAM-DAn representation to a predictive MBON representation when compared to sugar olfactory conditioning (Yamagata et al., 2015). Future experiments should thus try to decipher what PAM neurons are required for EDAP acquisition, and if they are the same or different from sugar conditioning.

Results indicate that PAM DA neurons are required at different time scales compared to TH-Gal4 neurons to influence EDAP in flies. DA release from PAM

neurons might be different from TH-Gal4 neurons. Future studies should endeavor to compare DA release from PAM and PPL1 neurons after exposure to ethanol. This is important because rescue of D1 receptor only in the MB and FSB, which are innervated by PAM and PPL1 respectively, is sufficient to show normal alcohol preference similar to wild type flies (See Figure 8.4). Furthermore, this information can give more insight into transition from NAA to EDAP in comparison to zonal PAM➔MB representation of EDAP acquisition. Transition from NAA to EDAP might therefore depend on the independent influences of alcohol on both the PPL1➔FSB and PAM➔MB neurocircuits. Neuroadaptive changes by alcohol could suppress the PPL1➔FSB microcircuit as the PAM➔MB neurons strengthen over time leading to EDAP (See Figure 10.5A). One possible way of testing this hypothesis is to test what happens to DA release over time in both PPL1 (or TH-Gal4) and PAM DA neuron after ethanol exposure. A caveat of using carrying out optogenetic/Fast Scan voltammetry experiments is that dissected brains would dead long before chronic ethanol experiments are carried out. One way to ameliorate this caveat is to expose fly to low doses of vaporized ethanol in vivo using the booz-o-mat (See Chapter 2) before carrying out optogenetic studies of dopamine release in dissected fly brains.

One last possibility affecting transition is that PPL1➔FSB and PAM➔MB circuits might be connected to each other. There seem to be a causal relationship between both circuits affecting the transition from NAA and EDAP. For example, silencing PAM neurons enhances NAA, while silencing PPL1 neurons somewhat enhances EDAP (see Chapter 8). Studies also indicate that MBONs project/connect to PPL1 neurons (Aso et

al., 2014; Mao and Davis, 2009). Therefore, the MB might suppress PPL1 DA neuron's inhibition of FSB activation as a fly learns to enjoy the intoxicating effects of alcohol (See Figure 10.5B below). If this hypothesis were true, it would indicate that although distinct microcircuits affect NAA and EDAP, connectivity between the FSB and MB circuits might create a causal relationship affecting transition from an aversive to an appetitive valence, similar to what is seen in mammals (Namburi et al., 2015). The FSB might thus be where EDAP memories are stored since result in chapter 8 show that the MB is required only during EDAP acquisition and not for retrieval (See Chapter 8). Therefore, input specificity on aversive DA neurons (PPL1 and possibly PPM3) to the FSB might act as a circuit switch for both aversive and rewarding/reinforcing valence similar to the VTA in mammals (Lammel et al., 2012). Identification of MBONs (possibly from the MB β-lobes) projecting to the PPL1 neuron would help to prove this hypothesis true.
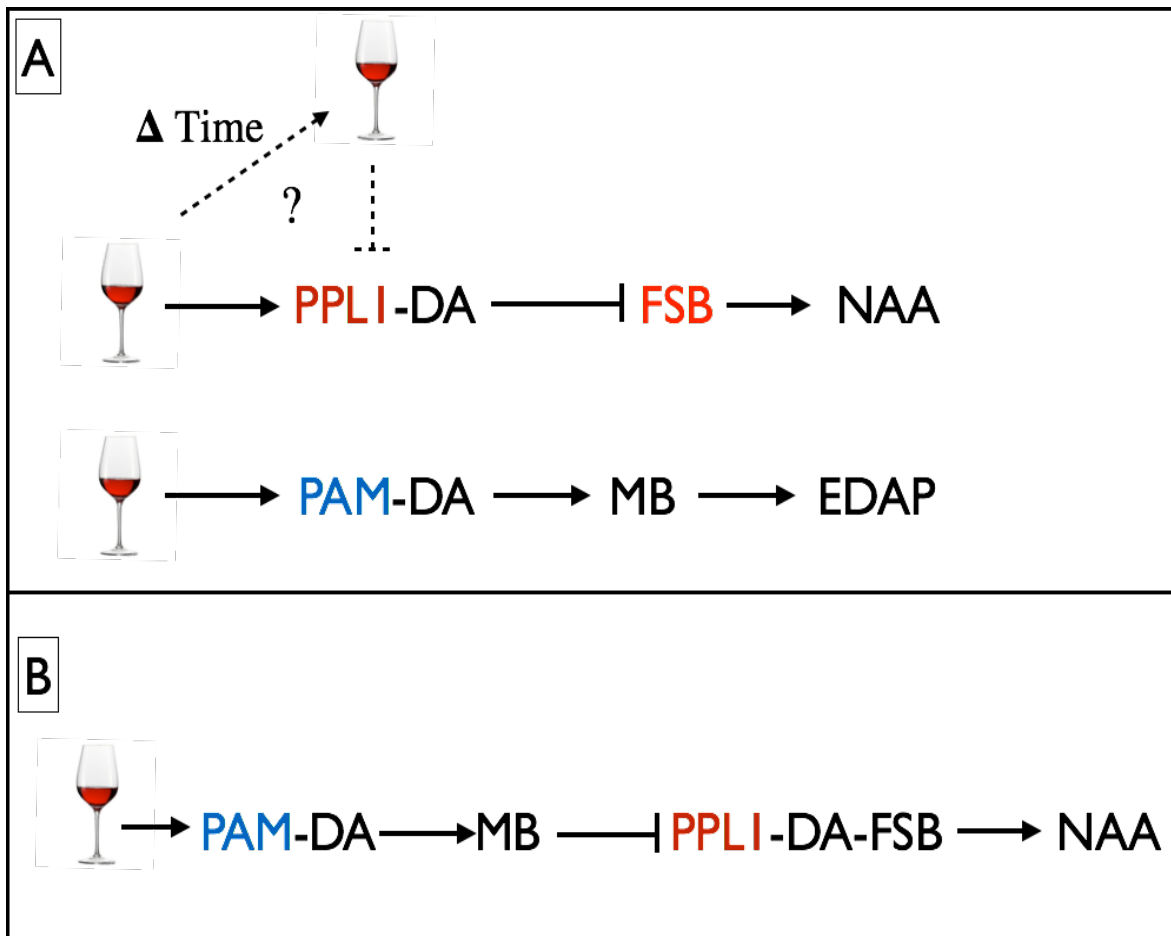
Figure 10.5. Hypothetical models suggesting that an independent or causal relationship between the MB and FSB affects transition from NAA to EDAP.

(**A**) Alcohol's effect on the PPL1➔FSB and the PAM➔MB microcircuits are independent of each other. When a naïve fly drink alcohol for the first time, PPL1➔FSB microcircuit is activated to promote NAA in flies. However through neuroadaptive changes, alcohol over time begins to suppress the PPL1➔FSB microcircuit preventing NAA, and strengthen the PAM➔MB promoting EDAP in flies.

(**B**) Second model suggest connectivity between the MB and FSB affects transition from NAA to EDAP. As a fly begins to learn to enjoy the pharmacological effects of alcohol, the PAM➔MB microcircuit suppress PPL1's inhibition of the FSB causing a gradual transition from aversion to indifference, and then to EDAP.

Alcoholism from flies to humans.

My dissertation shows that *Drosophila* treat intoxicating levels of alcohol the same way AUD patients do. Like humans, naïve flies would initially avoid alcohol because they are aversed to its taste. However with each exposure, flies gradually gain preference for alcohol and continue to drink despite its taste, and side effects such as motor incoordination. Eventually, flies begin to build up tolerance, they increasingly self-administer alcohol to gain the same initial rewarding feelings, and would seek out alcohol despite potential harm (i.e. electric shock, quinine, (Devineni et al., 2009; Kaun et al., 2011)) to themselves. Like humans, ethanol is a positive reinforcer and flies develop dependence for alcohol.

Like humans, flies also experience negative symptoms of withdrawal when alcohol is unceremoniously taken away. Flies experiencing withdrawal have lowered threshold for seizures and exhibit more seizures than naïve flies (Ghezzi et al., 2011). During withdrawal, flies exhibit cognitive impairment in learning that is only restored when alcohol drinking is resumed (Robinson and Atkinson, 2013). Flies also show relapse-like behavior and would immediately begin drinking at the same levels (without the gradual increase in preference) observed before the forced period of abstinence (Devineni et al., 2009). Therefore, flies to some extent can clinically represent the human condition and symptoms of alcohol-use disorders.

For decades, investigators studying the relationship between midbrain DA activity and addiction have established that increased DA neuron activity is required for reward-related processes (Schultz, 1997). For instance, single unit recordings in primates

performing an operant task showed that putative DA neurons are phasically excited (i.e. activated) when unexpected rewards (i.e. drugs, food) are presented (Schultz, 1997). This led to an alluring model suggesting that DA neurons activation in the VTA encodes reward prediction errors, which is the difference between an expected reward and the actual outcome (Lammel et al., 2014). The removal of an expected reward had the opposite effect on DA neuronal activity with silent periods associated with negative reward prediction errors. Thus, DA neurons fire when an unexpected reward is presented. My work with flies and recently works in mammals reveal that distinct dopaminergic neurons can be activated by aversive stimuli. For acute ethanol concentrations as low as 5mM was able to activate/stimulate dopamine release from DA neurons required for naïve alcohol avoidance in flies (See Chapter 8). Mammalian studies recently reached the same conclusion as it was discovered that anatomically distinct subsets of dopamine neurons in the VTA are required for reward and aversion in mice (Lammel et al. 2010). Also, aversive and stressful events can excite VTA DA neurons just as rewarding stimuli do (Brischoux et al., 2009; Lammel et al., 2014). Taken together, my studies in flies indicate that like mammals, there are two 2 functionally and anatomically distinct dopamine circuits required for aversion and reward.

Since alcohol activates but aversive and appetitive DA neurons, it suggests that gradual transition from NAA to EDAP might have to do with continued conflict between DA inputs into structures required for aversion (FSB) and reward (MB). It has been proposed that addiction is a type of pathological associative memory that is produced by

the overactivation of a reward pathway (Robinson and Atkinson, 2013; Nestler, 2002). If my hypothesis of an initial struggle is true, it would suggest that naïve avoidance observed in flies is likely due to the aversive taste of alcohol and that struggle with rewarding DA input would not occur since the rewarding effects of alcohol are not yet learned. That is, the first time a naïve fly drinks alcohol, it might decide not to approach alcohol before the euphoric effects of alcohol is felt. However, over time flies gradually learn to associate the taste of ethanol with its post-ingestive effects and the struggle between the aversive and appetitive DA circuits commence. Since the MBs are required for acquisition of EDAP, it would suggest that learned alcohol reward occurs in the MB through the process of trace conditioning. Trace conditioning occurs when the stimulus or action terminates well before the reinforcing effect is observed. These phenomena are also observed in mice. For example, infant rats exhibit aversive learning to ethanol's orosensory effects, but show positive reinforcement to its post-ingestive effects (Pautassi et al., 2009). Acute exposure to other drugs of abuse such as nicotine in rats also results in short-term conditioned place aversion that switches to longer-lasting conditioned place preference (Laviolette and van der Kooy, 2004).

Further experiments testing these hypothesis is required to understand how transition from NAA to EDAP occurs. Since role in of DA in aversive and reward learning is similar conserved in flies and mammals, studying the effects of alcohol or other drugs of abuse on gene expression in these DA circuits can be easy carried out, and could accelerate our ability in finding treatment to ameliorate the negative reinforcing effects of alcohol in AUD patients.

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Conclusion

Alcoholism research in *Drosophila* has focused on the development and characterization of fly behavioral assays that are already well established in mammalian model systems. This is necessary because *Drosophila* has only more recently become an alcoholism model system. Despite the differences that exist between humans and flies, the conservation and face validity of behavioral responses to ethanol has to this point been impressively similar (See chapter 3). Not only are the adaptive responses of alcohol consumption conserved, I show evidence of similar interactions between ethanol and the reward-and-learning mechanisms in flies and mammals. That is the approaches I employed in flies to validate Rsu1's role in alcohol consumption and reward were predictively valid and translatable to human RSU1 phenotypes (See chapter 4). I also show that the Rsu1/Rac1/LIMK/Cofilin/Actin signaling pathway is mechanistically valid in flies and humans to affect learning and reward circuits in the brain to not only alcohol but to other drugs of abuse in mammals (loss of Rsu1 in flies also leads to resistance to both nicotine and cocaine) (Dietz et al., 2012; Kiraly et al., 2010; Offenhauser et al., 2006). The reason why ethanol responses are so tightly conserved between mammals and invertebrates might be because the lists of functionally relevant ethanol/drugs of abuse targets include some evolutionarily ancient cellular mechanisms. I show that dopamine, an evolutionarily ancient cellular neurotransmitter once taught to be required for only aversive memories in flies, controlled both aversive and appetitive valences in different brain circuits as observed in mammals (Robinson and Atkinson, 2013). In conclusion, my dissertation proves that Drosophila has face, mechanistic, and predictive

validity for studying genes, behaviors, and neuronal circuits that are regulated by alcohol, and can help accelerate our understanding of their functional relevance in human addiction.

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