BRAIN MOLECULES UNDER THE INFLUENCE: INTRACELLULAR REGULATION OF BEHAVIORAL RESPONSES INDUCED BY ETHANOL

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By

Raniero Leonette Peru y Colón de Portugal

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DEDICATION

To my wonderful and beloved mother for her unconditional love, patience, and support. My eternal gratitude goes to you, mother. Thank you for giving me the greatest gift anyone could receive…the gift of life! Without you and God, this dissertation would have never been possible. To all the exemplary and dignified men and women of honor, my forefathers, thank you for transmitting me the moral values necessary to live with integrity and rectitude, I am humbled to honor your memory through this dissertation. To my friends, family, and all those who believed in me and encouraged me to purse my goals, dreams, and aspirations.

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A REFLECTION ON SCIENCE

On the 3rd of August 1492, Christopher Columbus and his men embarked on their first voyage across the Atlantic on route to discover and explore new lands, which later came to be known as the Americas. Since the beginning, this voyage was filled with hardships and unknowns. Indeed, it was a journey his contemporaries believed was not possible, although 519 years of history have proven these beliefs were inherently wrong. When studying the history of my forefather, I can see many parallels with the scientific profession. Though as scientists or explorers we often stumble upon shores of lands previously traversed by others, the real discovery may not come from being the first to set foot on these new lands, but rather, from having the vision and determination to overcome hardships and unknowns. To succeed, one must have a sense of adventure and the desire to delve into the study and exploration of the wonders of these previously traversed lands and bring them to the attention of the scientific community and the world.

ABBREVIATIONS

AC	Adenylase Cyclase
ADH	Alcohol Dehydrogenase
AKT	Protein Kinase B
AMPAR	α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor
ANOVA	Analysis of Variance
AP-2	Assembly Protein Complex-2
Arf6	Adenosine Diphosphate Ribosylation Factor 6
Arfaptin	Adenosine Diphosphate Ribosylation Factor-Interacting Protein
Arfip	Arfaptin
ARNO	ADP-Ribosylation Nucleotide Exchange Factor
aru	arouser
AUTS2	Autism Susceptibility Candidate 2
AUD	Alcohol Use Disorder
BACE1	Beta-Site Amyloid Precursor Protein Cleaving Enzyme 1
BDNF	Brain-Derived Neurotrophic Factor
BK	Big Potassium Channel
BFA	Brefeldin A
CA	Constitutive Active
CAFE	Capillary Feeder
CAM	Cell Adhesion Molecule
cAMP	Cyclic Adenosine Monophosphate
CDE	Clathrin-Dependent Endocytosis

CGN	Cerebellar Granule Neurons
CHO	Chinese Hamster Cells
Chpd	Cheapdate
CIE	Clathrin Independent Endocytosis
CNS	Central Nervous System
CPP	Conditioned Place Preference
dAlk	*Drosophila* Anaplastic Lymphoma Kinase
dLmo	*Drosophila* LIM-Domain Only
DN	Dominant Negative
DNT	*Drosophila* Neurotrophin
DoR	Dopamine Receptor
DSM-IV	Diagnostic and Statistical Manual of Mental Disorders
EEA1	Early Endosome Antigen 1
ELAV	Embryonic Lethal Abnormal Vision
ERK	Extracellular Signal-Regulated Kinase
EPS8	Epidermal Growth Factor Receptor Kinase Substrate 8
FAS	Fetal Alcohol Syndrome
FITC	Fluorescein isothiocyanate
FOXO	Forkhead Box Protein O
GABA	Gamma-Aminobutyric Acid
GABRA	GABA Receptor Alpha
Gal80ts	Gal80-Temperature Sensitive
GAP	GTPase Activating Proteins
GDP	Guanine Diphosphate
GEF	Guanine Nucleotide Exchange Factor

GFP	Green Fluorescent Protein
GIRK	G Protein-Coupled Inwardly-Rectifying Potassium Channel
GTP	Guanine Triphosphate
hangover	*hang*
hOP-1	Human Osteogenic Protein-1
IGF	Insulin Growth Factor
InR	Insulin Receptor
IPC	Insulin Producing Cell
IRS-1	Insulin Receptor Substrate-1
JWA (or ARL6ip5)	ADP-Ribosylation-Like Factor 6 Interacting Protein 5
KCNMA1	Potassium Large Conductance Calcium-Activated Channel, Subfamily M, Alpha Member 1
KCNX	Potassium Voltage-Gated Channel
Km	Michaelis Constant
Kra	Krasavietz
L1	L1 Cell Adhesion Molecule
LOR	Loss-Of-Righting
LTD	Long-Term Depression
MAPK	Mitogen-Activated Protein Kinase
MBs	Mushroom Bodies
mEPSC	Miniature Excitatory Post-synaptic Current
mys	Myospheroid
NAc	Nucleus Accumbens
NMDA	N-Methyl-D-Aspartic Acid
NMJ	Neuromuscular Junction

NPY	Neuropeptide Y
NPF	Neuropeptide F
NRK	Normal Rat Kidney
NS	Nervous System
PA	Phosphatidic Acid
Par-1	Polarity Kinase-1
PDK1	Phosphoinositide-Dependent Kinase-1
PEt	Phosphatidylethanol
PLD	Phospholipase D
PI3K	Phosphoinositide-3-Kinase
PIP_2	Phosphatidylinositol 4,5-Bisphosphate
PIP5K	Phosphatidylinositol-3-Phosphate 5-Kinase
PKA	Protein Kinase A
PKB	Protein Kinase B
POR1	Partner of Rac1
PSD	Post-Synaptic Density
PTEN	Phosphatase and Tensin Homolog
Rac1	Ras-Related C3 Botulinum Toxin Substrate 1
Rho	Ras-Homology
RTK	Receptor Tyrosine Kinase
scb	Scabrous
SEM	Standard Error of the Mean
Shaw2	Shaker Cognate W Protein
SHC	Src Homology 2 Domain Containing Transforming Protein 3
Shi	Shibire

S6K	S6 Kinase
SK6-P	S6 Kinase-Phosphorylation
Slo-1	Slow Poke-Gene 1
ST-50	Time to 50% Sedation
Syn	Syntaxin
SYX1A	Syntaxin-1A
Tao	Thousand and one Protein
TfR	Transferrin Receptor
TrkB	Tyrosine Kinase B Receptor
TOR	Target of Rapamycin
TM	Transmembrane
TRH	Thyrotropin-Releasing Hormone
TRP	Transient Receptor Potential Channel
TSC	Tuberous Sclerosis Complex
UAS	Upstream Activation Sequence
whir	white rabbit
WT	Wild-type

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Alcohol abuse is a devastating condition affecting millions of individuals. Regulation of insulin receptor (*InR)* signaling is critical for ethanol–induced responses and consumatory ethanol behavior. However, the precise intracellular mechanisms regulating *InR* signaling, which in turn, affect ethanol-induced behaviors remain unknown. I describe an *InR*/*Arf6*/*S6K* pathway that controls acute ethanol responses in *Drosophila*. I show that *Arf6* mutants are hypersensitive to ethanol's intoxicating effects, and that *Arf6* is specifically required in the adult nervous system to regulate naïve ethanol sensitivity. While *Arf6* functionally integrates activated *Rac1* to the *InR* signaling, neuronal *S6K*, an *InR* effector, is a key mediator of *Arf6*-dependent regulation of ethanol-induced behaviors. Ethanol vapor concentrations that produce moderate sedation increase *S6K*-P, while doses that confer total sedation completely abate *S6K*-P. *Arf6* mutants are completely devoid of neuronal *S6K*-P at baseline, suggesting that lack of *S6K*-P.

pre-sensitizes *Arf6* mutants to the intoxicating effects of ethanol, and thus sedate at low physiologic ethanol concentrations. Because *Arf6* has been implicated in receptor-mediated endocytosis, and signal transduction pathways are largely regulated by receptor trafficking, I propose a model in which *Arf6* regulates *InR* signaling via endocytosis to control behavioral ethanol responses.

Soon after joining the Rothenfluh laboratory in the Fall of 2008, I began studying the relationship between Ras-Homologous GTPase Activating Protein 18D (*RhoGAP18B)*, Adenosine Diphosphate Ribosylation Factor 6 (*Arf6*), Adenosine Diphosphate Ribosylation Interacting Factor Interacting Protein 2 (*Arfaptin2* in mammals, but since flies only have one identified *Arfaptin,* I will refer to it as *Arfip)*, and Ras-Related C3 Botulinum Toxin Substrate 1 (*Rac1)*. In broader terms, how a *Rho-to-Arf* signaling controls behavioral responses to ethanol. Previously, Rothenfluh and colleagues (2006) had demonstrated that *white rabbit* (*whir*) mutants show disruptions in *RhoGAP18B*, and display strong resistance to ethanol sedation.

Supporting my focus on *Rac1*, *RhoGAP18B* protein, which belongs to the *Rho* subfamily of the *Ras* super family of small GTPases, regulates *Rac1* activity in vitro via its *GAP* domain in vitro. A modifier screen was conducted to further define the molecular pathway through which *RhoGAP18B* mediates behavioral sensitivity to ethanol. This screen resulted in the identification of *Arf6* (also known as *Arf51* in *Drosophila*) pathway mutations, which I have characterized since

initiating my dissertation, and will describe in the results section. Initially, these Arf6 pathway mutations were isolated due to their ability to interact genetically with the strong *whir*^{3} allele of *RhoGAP18B*, and further experiments show that they also modify the ethanol resistance of *RhoGAP18B* mutants.

Although many reports shed light on the role of *Arf6* in terms of signal transduction and actin regulation (D'Souza-Chorey et al., 1997), I knew little about how mechanistically *Arf6* could contribute to ethanol responses. Of the *Arf* GTPases, *Arf1* and *Arf6* have been the best studied (Biou et al., 2010; Nie et al., 2003; Donaldson and Jackson, 2011), and although the mammalian *Arf6* shares about 97% amino acid identity with the *Drosophila Arf6,* its functional characterization has been studied almost exclusively in cell culture systems, while its *in vivo* function remains to be elucidated. Studying *Arf6* in mammals has proven difficult given that *Arf6* mutant mice die embryonically due to liver defects (Suzuki et al., 2006), and to date, no study has been published using conditional Arf6 knockout mice.

While pondering on what sets of experiments to perform, a report by Rankovic et al. (2009) showed that *Arf6* endocytoses and recycles the mu-opioid receptor in synergy with Phospholipase D *(PLD)* in rat-derived neuronal cultures. This data was interesting because it links Arf6 to the regulation of mu-opioid receptor trafficking, but more importantly to addiction. Because no mu-opioid receptors have been identified in flies, I set out to identify a receptor that could be

regulated by *Arf6* in flies. If successful, I would be able to study how *Arf6* regulation of signal transduction controls ethanol-induced behaviors. In more general context, I could begin to elucidate how extrinsic and intrinsic signals are integrated in the nervous system to control behavior. I wanted to understand how addiction-relevant extracellular signals are regulated intracellularly, and investigate how, and which signal transduction molecules could orchestrate ethanol-induced behaviors.

Corl and colleagues showed that *InR* mutants are sensitive to sedating effects of ethanol (Corl et al., 2005). While there were a few reports from the 1990s suggesting an involvement of Arf6 in InR signaling, however, other reports did not find a requirement for Arf6 in InR signaling regulation. The fact that *Arf* GTPases had been ascribed many important roles in actin cytoskeleton dynamics (Campa et al., 2008), neuronal plasticity (Jaworski et al., 2007), growth and development (Suzuki et al., 2006), and membrane trafficking (Rankovic et al., 2009; Donaldson et al., 2009 and 2011), did not make my task of finding a receptor any easier.

Because Arf6 plays a role in neuroendocrine function, and insulin signaling is critical for this process, I was intuitively compelled to explore whether Arf6 would mediate InR signaling, particularly because the ethanol sedation phenotype of *InR* mutants was reminiscent of Arf6. After further reviewing the literature, various lines of evidence finally convinced me pursue this hypothesis –

driven approach. First, the *Drosophila* cytohesin *Steppke* (Arf6-*GEF*) functions in *InR* signaling (Fuss et al., 2006), which had been previously shown to play a major role in regulating ethanol-induced sedation (Corl et al., 2005).

Phospholipase D (*PLD*) is an effector of *Arf6*, which activates *InR* effector Phosphatidylinositol-3-Phosphate 5-Kinase *(PIP5K),* through its secretion of Phosphatidic acid (*PA*)(Cockcroft, 1996; Van den Bout and Divecha, 2009). Once activated, *PIP5K* in turn activates a phosphoinositide cascade leading to downstream Protein Kinase B Phosphorylation (*AKT-P*) and S6 Kinase Phosphorylation *(S6K-P)* (O''Neil et al., 2009). These data was interesting given that both *PLD* and *PIP5K* are known effectors of *Arf6* (Funakoshi et al., 2011). Since initiating this project, we have uncovered an essential role for *Arf6* in acute ethanol behaviors through the regulation of the insulin receptor-signaling pathway, which will be introduced in the results section, and further delineated in the discussion section of this dissertation.

My doctoral work on the intracellular mechanisms that govern ethanol's intoxicating effects on behavior will be described. The present dissertation is divided in four main sections: 1) *Introduction*, 2) *results and methods, which include figures and figure legends and 3*) a *discussion* of the results. In the introduction, I will review the scientific literature on the regulatory mechanisms of

ethanol-driven behaviors performed in humans and other vertebrate species, while the central focus of this thesis is on *Drosophila* research.

In doing so, I will also highlight current issues and problems concerning the study of ethanol's direct and candidate targets, which affect behavioral responses to ethanol. In the result and methods section, I will describe my obtained experimental data and the methodology employed. In the discussion section, I will first illustrate on the initial part of the results dealing with neuronal *Rac1*, *Arfip,* and *Arf6*, which through a linear genetic pathway regulate acute ethanol sedation. Second, I will explain how *Arf6* GTPase may integrate the *Rho* to *InR* signaling to control behavioral ethanol responses. Third, I will illuminate on recent data showing that *Arf6* via neuronal *S6K* mediates behavioral sensitivity to ethanol. Moreover, I will propose a model in which Arf6 plays separable but intertwined roles in InR signaling and endocytosis, in order to regulate acute ethanol behaviors.

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Introduction

Genes, Molecules, and Behavior: Searching for Alcoholism's Targets

Alcohol has been one of the most widely consumed and abused substance for millennia, in part due to its pleasurable and disinhibitory effects at low acute intake levels, but also due to its addictive effects when consumed chronically. In the United States (U.S.) alone, more than 105,000 annual deaths are ascribed to alcohol use (McGinnis and Foege, 1999) and more than 20% of hospitalized patients have alcohol abuse among their list of illnesses (Diamond and Gordon, 1997). Alcohol dependence is a consequence of alcohol use, and it is considered as a primary and chronic disease afflicting roughly 7% of the U.S. adult population (Diamond and Gordon, 1997). Although epidemiological studies show a greater prevalence for alcoholism in males than females of distinct ethnic groups, its negative correlation with education and socio-economic status, but positive correlation with marital problems is independent of gender and ethnicity (Bucholz, 1992; Helzer and Pryzbeck, 1988; Long et al., 1998; Helzer et al., 1991).

Alcohol consumption is part of the cultural heritage and social experience of individuals in many nations, yet its adverse consequences tremendously impact the health (Kessler et al., 1994) and wealth (Volpicelli, 2001) of its citizens and society as a whole (WHO, 2004). While alcohol consumption is a pre-requisite for addiction, alcohol dependence is a hallmark of alcoholism (Grant et al., 2009),

and it is characterized by continued alcohol consumption despite of adverse consequences related to the individual's health and social status (American Psychiatric Association, 1987). The basis of alcoholism is complex, and is at least 39%-60% hereditary according to adoption and twin studies (Spanagel, 2009; Bohman et al., 1984; Cloniger et al., 1981; Heath et al., 1997).

Human genetic studies have revealed a high correlation between alcoholism and genetic variation in the $GABA_A$ α2-receptor subunit (GABRA2) (Edenberg et al., 2004; Enoch and Goldman, 1999; Lappalainen et al., 2005), and also highly correlated to alcoholism is linkage in the $GABA_A$ α6 receptor subunit (GABRA6) region of chromosome 5 of Finnish and Native American subjects (Radel et al., 2005). Other studies have found differences in ethanol behavioral responses across ethnic groups (Chan et al., 1986; Agarwal et al., 1981; Goedde et al., 1979), perhaps reflecting the extent to which distinct cultures embrace alcohol consumption as part of their traditions.

Several well-defined Native American populations have been the focus of intense genetic studies given their extremely high rates of male alcoholism. Because alcoholism is intrinsically polygenic and environmentally complex, individuals in these populations are considered well-suited experimental subjects given their genetic and cultural homogeneity (Lander and Schork, 1994). Indeed, linkage analysis in Southwestern Native Americans uncovered alcohol dependence risk linkage loci on chromosome 11p, proximal to alleles of the

tyrosine hydroxylase (*TH*) and DRD4 dopamine receptor, and on chromosome 4, proximal to the GABA β1 receptor gene subunit (Long et al., 1998).

Several lines of evidence indicate that the level of response to sedating ethanol doses is predictive of alcoholism (Schuckit et al., 2004). According to human studies, a 20 year old individual displaying acute resistance to ethanol intoxication is four times more likely to become an alcoholic 10 years later compared to control subjects (Schuckit et al., 2004; Schuckit, 1994). This data suggests that acute ethanol responses are highly correlated with future alcohol consumption and addiction in human and rodent models. However, recent studies in flies have shown that naïve ethanol responses may not be indicative of alcohol consumption and preference, but that development of ethanol tolerance (or the acquired resistance to ethanol's intoxicating effects)(Kaun et al., 2012), may be a more accurate predictor of addiction-related ethanol behaviors (Devineni et al., 2011).

Research in humans has many limitations for elucidating molecular and genetic pathways, that is why research in other vertebrate and invertebrate model systems, as well as *in vitro* approaches have proven vital to achieve a better understanding of the molecular genetic underpinnings of alcohol addiction. Candidate ethanol effectors likely share common ethanol targets as upstream regulators, thus being key signaling mediators of ethanol's direct effects on membrane proteins, as well as major determinants of an organism's behavioral

sensitivity to ethanol. As it could be expected given ethanol's pleiotropic effects, ethanol can also affect additional signal transduction targets in a tissue specific manner, such as tyrosine kinase activity (Seiler et al., 2000), and the protein synthesis machinery (Berger et al., 2004; Lang et al., 2000). Understanding how acute alterations by ethanol on receptor signaling and endocytosis, as well as translational and post-translational mechanisms are correlated with mechanisms associated with alcohol addiction may help in the development of therapeutic strategies for early stages of alcoholism.

Common themes among ethanol's binding pockets have been identified (Harris et al., 2008; Mihic et al., 1997; Wick et al., 1998; Lobo et al., 2004; 2008). Most of ethanol's binding pockets are water containing and have two or more helices with amphipathic surfaces (Harris et al., 2008; Mihic et al., 1997; Wick et al., 1998). Another commonality is receptor subunit composition, which determines the efficacy of ethanol's effects, and yet another is that the ethanol appears to specifically target the transmembrane (TM) and extracellular domains of proteins (Harris et al., 2008; Perkins et al., 2008). Despite many advances in the field, many challenges remain including understanding how receptor composition functions in determining receptor sensitivity to ethanol effects, determining whether ethanol's binding pockets can physically accommodate ethanol molecules and cause the displacement of water for ethanol at low millimolar ethanol concentrations.

To establish a molecule as a directly binding ethanol target, various requirements have been proposed (Harris et al., 2009). First, the *in vitro* functional alteration of a protein by exposure to physiologically relevant ethanol doses (5-150 mM) must be shown. Second, ethanol's binding affinity must be altered as a result of experimentally generated mutations in a protein's amino acid sequence. Third, there should be consistency between *in vivo* and *in vitro* data resulting from manipulation of the protein of interest. Finally, evidence for the presence of an ethanol molecule within the target must be provided through atomic structure analysis. A key question that these approaches have not yet answered is how exactly do human polymorphisms in $GABA_A$ subunits alter ethanol-induced behaviors and contribute to alcoholism.

Given how little is known about ethanol's direct effects on specific targets, as well as limitations in human studies, it is argued herein that studying genetic alleles and relevant signal transduction pathways in genetically amenable and simplistic systems, such as *Drosophila* melanogaster may provide greater insight into the molecular genetic basis of alcohol addiction. Research in *Drosophila* has many advantages over mammalian models. These involve sophisticated forward genetic screening capabilities, reduced financial cost, high number of offspring and generations that can be produced given their short life cycle and increased fecundity. In addition, their small size enables the researcher to maintain thousands of fly mutant stocks in the laboratory.

Aside of these classical advantages, the *Drosophila* genome is highly homologous to the mammalian genome. Although mammals have roughly three times the number of genes than flies, they both have similar number of gene families (Holland, 2003). In addition, at least 75% of gene orthologues associated with human disease can be found in flies (Chien et al., 2002). These alleles also share a high degree of functional similarity, indicating that flies can be used as a genetic model to investigate human disease (Kaun et al., 2012).

Perhaps most perplexing is that despite of having approximately 300,000 neurons in the entire nervous system, recent behavioral genetics developments have succeeded in showing that flies display complex behaviors usually ascribed to higher order organisms such as mammals, which have billions of neurons. For instance, flies are able to integrate sensorimotor information to adjust and navigate in their spatial environment (Pick and Strauss, 2005). Flies can also form associations between reinforcing and aversive stimuli in learning and memory tasks (Quinn et al., 1974; Davis, 1993), and are able to make choices that range from female oviposition (Joseph et al., 2009) to choosing over drinking ethanol-containing solutions versus sucrose (Kaun et al., 2011). Flies also show social behaviors such as fighting (Chen al., 2002), courtship (Greenspan and Ferveur, 2000), and exhibit addiction-like behaviors in response to environmental manipulations such as female sexual rejection (Ophir-Shohat et al., 2012).

Forward-genetics (i.e. going from phenotype to genes) remain as the favored approach in *Drosophila,* although subsequent to the establishment of genetic transformation (Rubin and Spralding, 1982), reverse genetic approaches (i.e. going from genes to phenotype) have also been employed. Forward genetic approaches in particular, have yielded alleles that affect ethanol-driven behaviors in flies and mice, and polymorphisms of their human homologues have been linked to alcohol sensitivity (Lasek et al., 2011b; Kaun et al., 2012). In a study using humans, mice, and flies, Lasek et al (2011) showed that *Drosophila* anaplastic lymphoma kinase (d*Alk*) regulates ethanol sensitivity in flies, and then showed that *Alk* mutant mice phenocopied *dAlk* mutants (Lasek et al., 2011b). Because *Alk* contributes to human disease, the authors sequenced the human *Alk* gene for 348 subjects that previously showed sensitivity to an oral ethanol challenge, and found that homozygous subjects for the minor allele rs17007646 showed ethanol resistance in two independent behavioral measures (Lasek et al., 2011b). Similarly, Riley et al. showed that the human gene ZNF699, which is related to the ethanol tolerance *Drosophila* allele *Hangover* (*Hang)*, is associated with alcohol dependence in a sample of human sibling genetic study (Riley et al., 2006).

Some success has also been obtained with meta-analysis of human genome-wide data, which has uncovered mutations in disease-associated genes, which strongly correlate with alcohol drinking behaviors in mice and ethanol

response sensitivity in flies, such as the gene *autism susceptibility candidate 2* (*AUTS2*) (Schumann et al., 2011). Taken together, this data indicates that results obtained in *Drosophila* studies are relevant for human disease, and that *Drosophila* alleles isolated through forward genetic approaches may guide reverse genetics research in performed in mammals (Kaun et al., 2012).

As in humans, lower ethanol doses produce hyper-activation in flies, which can be assessed by a tracking device that measures the speed of locomotion elicited as pharmacologically relevant ethanol levels increase, while higher ethanol doses confer loss-of-righting (LOR) or sedation, that is the inability to regain upright posture after falling to the floor's surface (Rothenfluh et al., 2006; Corl et al., 2009; Eddison et al., 2011). It is relevant for addiction research to study ethanol-induced LOR, as genetic manipulations that produce reductions in sedation have been shown to increase self-administration in mammals (Thiele et al., 1998).

Assays to Study Ethanol Induced Behaviors in Drosophila

Assays that extract and systematically measure behavioral features of alcohol addiction have continued to evolve and it is now possible to study naïve ethanol responses and more complex behaviors like self-administration and conditioned place reference (CPP) in flies (Kaun et al., 2011). Genes that regulate ethanol-induced behaviors in flies and mammals have been shown to

greatly overlap, although many genes identified in flies still require confirmation in mammalian models.

Flies, like mammals, display acute ethanol responses, and the level of response to a naïve ethanol exposure are correlated with ethanol drinking in mammalian systems and alcoholism in humans (Schuckit, 1994). Acute sensitivity to ethanol doses that induce motor dysfunction is linked to decreased risk for alcohol abuse (Wolf et al., 2002; Kurtz et al., 1996; Morean and Corbin, 2010). To measure naïve responses to ethanol, flies are delivered doses of ethanol vapors mixed with air at ratios that can be regulated in order to monitor the influx of ethanol received (Wolf et al., 2002).

Ethanol-Induced Behaviors in Drosophila: Naïve Ethanol Sensitivity

Innately, flies display negative geotaxis, thus preferring to be at the top of a column. Acute ethanol sensitivity can be studied using an apparatus known as “the inebriometer”, which is designed as a column with multiple baffles. In this assay one can measure the time it takes for flies to hit the bottom of the column as they lose motor control and continue to fall through the baffles (Scholz et al., 2000). The “booze-o-mat” is another an apparatus to measure ethanol responses in flies, and delivers ethanol/air doses to flies, which have been placed in transparent plastic tubes. The ST-50 or the time it takes for 50% of flies in any

given tube to reach sedation has been used as a measure to study behavioral sensitivity phenotypes (Rothenfluh et al. 2006).

Ethanol Tolerance Behavior

Tolerance is a hallmark of alcoholism according to the Diagnostic and Statistical Manual of Mental Disorders (DSM-IV) (American psychological Association, 1994). Two forms of tolerance have been studied. Rapid tolerance is essentially an acquired resistance (or reduced sensitivity) to the effects of ethanol after a brief exposure, and chronic tolerance is acquired after prolonged exposure to lower ethanol doses that fail to induce overt ethanol phenotypes. Unlike chronic tolerance, rapid tolerance does not require protein synthesis (Berger et al., 2004), and its acquisition is not metabolic in nature (Berger et al., 2004; Cowmeadow et al., 2005; Scholz et al., 2004), indicating that rapid and chronic tolerance are both acquired via distinct mechanisms, and perhaps regulated in distinct subpopulations of neurons. In flies, both forms of tolerance can be assessed in either the inebriometer or booze-o-mat, and it can be quantified either as a difference in the number of sedated flies between the first and the second (or repeated) ethanol exposures (Urizar et al., 2007), latency of intoxication (Dzitoyeva et al., 2003; Devineni et al., 2011) or recovery time after LOR (Berger et al., 2004; Cowmeadow et al., 2005).

Ethanol Self-Administration and Conditioned Ethanol Preference (CPP)

Recently new paradigms have emerged that made it possible to assess how far flies are willing to go to voluntarily consume ethanol. Much more is known about the genes that regulate naïve ethanol responses and to a lesser extent their involvement in ethanol tolerance. In a two choice assay, flies prefer ethanol-containing food over regular food. In this paradigm, flies are given the choice to drink ethanol + sucrose, over sucrose alone via capillary tubes. When the bitter compound quinine is added to the ethanol-containing food, the initial response is aversion, but flies overcome the aversive taste of quinine to consume similar volumes as in non-aversive conditions (Ja et al., 2007; Devineni and Heberlein, 2009). Similarly, when flies receive a cue paired to an intoxicating ethanol dose, flies endure an electric shock to obtain the ethanol-paired cue (Kaun et al., 2011).

A conditional ethanol preference test was recently created to determine whether or not flies display behavior indicative positive reinforcement by ethanol exposure (Kaun et al.2011). In this test, flies are exposed to two distinct odors. for 10 minutes sequentially, and one of the two odors is paired with a moderately intoxicating ethanol vapor dose. Subsequently, flies choose between the two odors and preference for ethanol is determined based on the choice flies make between the ethanol-paired odor and the non-paired odor. Contrasting the conditioned ethanol preference used in mammalian models, this assay enables the ethanol dosage received by flies to be regulated by the investigator, and the

ethanol-paired odor rather than ethanol itself, is used as the rewarding stimulus during preference portion of the assay (Kaun, Devineni, and Heberlein, 2012).

Targets of Ethanol-Related Behaviors: Evidence from In Vitro and In Vivo Systems: 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 substrate specificity and catalytic efficiency (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 distinct behavioral sensitivities to ethanol (Agarwal et al., 2000). However, studies in larvae (David et al., 1976) and flies (Singh and Heberlein, 2000; Cohan and Graf, 1985; Morozova et al. 2007) have revealed that although ADH alleles are important for the toxic effects of ethanol, ADH does not regulate ethanol-induced LOR.

Adenylyl cyclases (ACs) belong to a distinct class of enzymes that alter ethanol-induced behaviors (Kim et al., 2011), with distinct isoform variation showing distinct sensitivity to ethanol-induced cAMP production (Yoshimura and Tabakoff, 1995). Of these, AC7 shows the strongest sensitivity of the AC isoforms, with AC3 being completely insensitive, and AC2, AC5, and AC6

showing moderate sensitivity (Yoshimura & Tabakoff, 1995). Studies in *Drosophila* and mice have shown that AC regulates behavioral responses to ethanol (Eddison et al., 2011; Maas et al., 2005; Park et al., 2000). Moore et al. (1998) found that *cheapdate* mutants, an allele of the a cAMP-activating neuropeptide encoding gene *amnesiac*, show sensitivity to ethanol sedation, which can be rescued by decreasing protein kinase A (PKA) levels either genetically (Thiele et al., 2000) or pharmacologically (Moore et al., 1998). The role of cAMP in ethanol responses has been validated in mammals (Wand et al., 2001) and in Zebrafish (Peng et al. 2009), where they show that increasing neuronal cAMP produces ethanol resistance, decreasing cAMP produces sensitivity to ethanol.

A study using AC chimeras identified two ethanol sites of action; the first being a 28 amino acid region on the N-Terminal C_{1a} domain, and the second is located within the C-terminal domain (Yoshimura and Tabakoff, 1995). Quite surprisingly, Kruse et al. showed that the second site of ethanol action shows structural resemblance to the amino acid target sequence of the *Drosophila* protein LUSH, a well characterized non-enzymatic ethanol target that is required for olfactory detection and avoidance of particularly high or toxic ethanol doses (Yoshimura and Tabakoff, 1995; Kim et al., 1998; Kruse et al., 2003).

Ion Channels

Inhibition of the glutamatergic N-methyl-d-aspartate (NMDA), and the enhanced function of γ-aminobutyric acid type A ($GABA_A$) at ethanol doses of 30-100 mM produces behaviorally relevant effects, or lower doses by longer chain type alcohols (Dildy-Mayfield et al., 1986; Harris et al., 2009). Mutating the NMDA receptor transmembrane (TM3) domain changed the sensitivity to ethanol exposure, and variation in ethanol sensitivity appears to be influenced by receptor subunit composition, since NR2B receptors display stronger sensitivity to the enhancing effects of ethanol (Peoples and Weight, 1995; Ronald et al., 2001).

More recently, Offenhauser et al. (2006) showed that while primary cultured neurons of wild-type mice exposed to ethanol display a gradual decline in NMDA receptor current, cultured neurons derived from Epidermal Growth Factor Receptor Kinase Substrate 8 (EPS8) knock-out (KO) mice, which show both resistance to ethanol sedation and increased ethanol preference in a two-bottle choice paradigm, have reduced ethanol-induced NMDA current deterioration (Offenhauser et al., 2006). These data underscores the possibility that NMDA receptors are potential ethanol targets (Moonat et al., 2010; Nagy, 2004; Smothers et al., 2001). Supporting this notion, A114 TM segments of the glycine receptor have been shown to be important for ethanol's effects on currents (Mihic et al., 1997; Wick et al., 1998; Lobo et al., 2004; 2008).

$GABA_A$ receptors have been of great interest to the alcohol field (Wick et al., 1998). Behaviorally, mice lacking α1 $GABA_A$ receptors show sensitivity to ethanol-induced sedation (Blednov, 2003), though some other studies have shown variation to ethanol sensitivity (Kralic, 2003), perhaps reflecting differences in genetic background. Interestingly, the α4 (or α6), β3, and δ subunits of $GABA_A$ receptors are activated by ethanol concentrations doses as low as 3 mM ethanol (Wallner et al., 2003), which is approximately 6 times less the legal blood alcohol level allowed for driving in most US states (17.4 mM). δ $GABA_A$ KO mice drink less ethanol in a two bottle choice paradigm (Boehm et al., 2004; Mihalek et al., 2001). Supporting this data, Rewal and colleagues (2009) demonstrated that selective knockdown α4 $GABA_A$ in the nucleus accumbens shell also reduced ethanol consumption and preference, again circumscribing the role of receptor subunit composition in ethanol responses.

In mice, $GABA_A$ receptors regulate behavioral ethanol responses and ethanol drinking (Wick et al., 1998), and although $GABA_A$ receptors are present flies, only $GABA_B$ receptors have confirmed roles in *Drosophila* ethanol-induced behaviors. $GABA_B$ mutant flies show behavioral sensitivity to the intoxicating effects of ethanol and a decrease in rapid ethanol tolerance (Dzitoyeva et al., 2003), indicating opposing roles of GABA receptors in acute and tolerance ethanol behaviors or perhaps reflecting distinct regulation of different ethanol-

induced behaviors in distinct neuronal populations containing these receptors (Kaun et al., 2012).

Ion channels whose function can be reliably altered by an *in vitro* ethanol dose of less than 100 mM are the Shaw 2 voltage-gated channels (Kv3.x, KCNX), inwardly rectifying K+ channels (GIRK, Kir3x), and the large conductance calcium activated channels (BK, slo-1, and KCNMA1) (Covarrubias & Rubin, 1993; Lewohl et al., 1999), which are implicated in development of ethanol tolerance (Liu et al., 2006; Wang et al., 2009). While ethanol's effects on these channels has been investigated, only the ethanol binding site of Shaw2 has been studied by means of amino acid mutagenesis, and findings of these experiments implicate the S4-S5 intracellular linker as a putative site for ethanol's inhibitory effects (Shahidulla et al., 2003).

Trp Channels

Recent research indicates that Trp channels are mediators of ethanol's effects on behavior. While ethanol potentiates the vanilloid Trpv1, it inhibits the cold-menthol receptor Trpm8 (Weil et al., 2005), indicating that ethanol has differential roles on Trp channels (Benedikt al., 2007). Ellingson et al (2008) showed that KO mice of the capsaicin receptor Trpv1 failed to show an aversive response to ethanol taste, while being more resistant to sedation than wild-type (Blednov and Harris, 2009). Because ethanol can enhance Trpv1 response to selective Trpv1 agonist, capsaicin and protons, while reducing the threshold for

heat-induced activation from 42° to 34° degrees (Hirota et al., 2003), it has been suggested that Trpv1 may function in the burning sensation of ethanol (Blednov and Harris, 2009).

Previous studies on Trpm8 showed that PIP_2 desensitizes TRpm8 via a Ca2+ dependent mechanisms (Rohacs et al., 2005; Liu and Qin, 2005) Phosphatidylinositol 4,5-bisphosphate (PIP_2) was recently shown to reduce ethanol's inhibitory effects on the cold menthol trpm8 receptor, but inhibiting PIP_2 re-synthesis with *PI3K* inhibitor, wortmannin does not affect ethanol inhibitory effects on Trpm8 (Benedikt et al., 2007). Although studies are lacking for other lipid kinases, it has been shown that the lipid membrane environment is a determinant of the cold sensing properties of Trpm8 (Morenilla-Palao et al., 2009).

Cell-Adhesion Molecules and the Integrin Signaling Pathway

Ethanol inhibits cell-cell adhesion by interfering with morphogenesis inducing activity of hOP-1 protein, at similar doses as those achieved during "social drinking" (Charness et al., 1994). Amino acid mutagenesis analysis revealed an ethanol-binding site in the cell adhesion protein L1, localized between the Ig1-Ig4 domains. Alcohols like butanol, octanol, azibutanol, and azioctanol (Arevalo et al., 2007) bind to this site, although Octanol (Wilkemeyer et al., 2000) blocks ethanol's inhibitory effects on L1. L1 can activate integrin-mediated neuronal migration to extracellular proteins via membrane trafficking

and mitogen-activated protein (MAP) kinase signaling (Thelen et al., 2002). Thus, uncovering downstream targets of either L1 or the integrin receptor, and exploring whether and how cell adhesion defects alter behavioral ethanol sensitivity to produce ethanol tolerance may yield a more precise understanding of ethanol's actions downstream of its target.

A study by Bhandari et al. (2009) implicated the integrin signaling pathway in ethanol-induced behaviors. The authors showed that *Drosophila* mutants for the β and α isoform, encoded by the genes myospheroid (*mys*) (MacKrell et al., 1988) and scabrous (*scb*) (Grotewiel et al., 1998), compared to their wild-type counterparts, exhibit strong sensitivity to acute ethanol sedation and increased rapid tolerance. *scb* plays a role in memory for ethanol reward. Interestingly, *scb* affects Notch signaling (Lee et al., 2000) which is critical for memory, particularly development of long-term memory. It is unclear whether Notch signaling participates in *scb* dependent ethanol reward memory (Kaun et al., 2012).

Transcription and Translation in Ethanol-Induced Behaviors

Gene transcription has also been recently 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 (Lasek et al., 2011a; Scholz et al., 2005). Though little is known their downstream targets, *anaplastic lymphoma kinase* (*dAlk*) is the only identified *dLmo* target to date (Lasek et al., 2011b). *hang* on the other hand,

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).

Translation initiation factor, *Krasavietz* (*kra*) mediates behavioral sensitivity to ethanol intoxication. When assayed in a set of ethanol behavior paradigms, *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). Of these, only chronic tolerance has been shown to require protein synthesis (Berger et al., 2004). Although pre-treatment with a protein synthesis inhibitor produced a strong decrease in sensitivity for ethanol intoxication, suggesting the existence of constitutively synthesized proteins that are required for acute ethanol responses (Kaun et al., 2012).

Growth Factors and Receptors

The epidermal growth-factor receptor (EGFR) signaling pathway modulates ethanol's intoxicating effects in *Drosophila*. A recent 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 (Pierce et al., 2001). Inhibiting ERK phosphorylation in zebrafish enhances ethanol sedation when exposed to doses that normally confer hyperactivity (Peng et al., 2009). 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). To further demonstrate the conserved role of EGFR in ethanol-related behaviors, Corl et al (2009) showed that feeding EGFR Inhibitors Erlotinib and Gefitinib, dramatically increased ethanol sedation in flies and decreased alcohol consumption in rats. It would be interesting to see whether polymorphisms in EGFR related genes turn out to regulate addiction-like behaviors or are found to correlate strongly with alcoholism.

Neuromodulators

Human and rodent studies have shown that ethanol causes many *in vivo* alterations such as reduction in dopamine receptors in alcoholics (Volkow et al., 2007), the activation (Gessa et al., 1985) and increased firing rate (Brodie et al., 1990) of dopamine neurons in response to low ethanol doses and the reduction of dopamine signaling receptor by chronic ethanol consumption (Lucchi et al., 1988; Hoffmann et al., 1977). Studies have shown that lack of dopamine receptors increases voluntary ethanol consumption (Bulwa et al., 2011), though others found that dopamine receptor 2 (DoR2) mutants decrease place ethanol

preference (Cunningham et al., 2000), while disrupting DoR1 impairs alcohol seeking behavior (El-Ghundi et al., 1998). These studies suggest that distinct DoRs may participate in distinct signaling pathways in different neuronal populations, which may account for differences in alcohol-driven behaviors.

In *Drosophila*, DoR signaling has been implicated in ethanol reward (Kaun et al., 2011) and arousal (Bainton et al., 2000; Kong et al., 2010; Kume et al., 2005) A recent paper by Kong et al. (2010) showed that a pair of DoR expressing ellipsoid body neurons plays a major role in regulating ethanol-induced hyperactivity. Interestingly, the synaptic immediate early gene and *Drosophila* homologue Homer, functions in the ellipsoid body neurons to regulate ethanol-induced sedation (Urizar et al., 2007), however, it remains to be established whether Homer mediates downstream DoR signaling.

Octopamine is a biogenic amine resembling norepinephrine, which appears to function in rapid, but not chronic tolerance or acute ethanol responses (Scholz et al., 2000; Berger et al., 2004). Neuropeptide Y and its receptors (NPY-Y1, NPY-Y2, and NPY-Y5) have been linked to alcohol drinking behaviors (Thiele et al., 2002), which either induced or enhanced by anxiety in NPY deficient mice (Hwang et al., 1999; Roy et al., 2002; Thorsell et al 1998). These KO mice show resistance to ethanol-induced sedation, while displaying increased ethanol consumption, which is increased with greater alcohol concentration (Thiele et al.,

1998; 2002). Conversely, transgenic NPY expression reduces ethanol consumption behavior (Thiele et al., 1998; Palmiter at al., 1998).

The *Drosophila* homolog of the mammalian Neuropeptide Y, Neuropeptide F (NPF) receptor signaling, in contrast to DoR signaling, mediates acute sensitivity to the intoxicating effects of ethanol (Wen et al., 2005). Although both NPY and NPF regulate ethanol-driven behaviors, NPF has not been implicated in stress responses or drinking. At least for NPY, these studies suggest that neuropeptides may function in environment-gene interactions underlying consumatory behaviors reminiscent of alcohol addition and alcoholism.

Mammalian neurotrophins like Brain Derived Neurotrophic Factor (BDNF) have been demonstrated to be essential for nervous system function and addiction related-behaviors. BDNF can activate tyrosine receptor kinase receptor B (TrkB) and P75, although less is known about the latter. Haplodeficient BDNF mutants show increased ethanol preference (Moonat, 2010) Manipulations leading to reductions in TrkB signaling did not add to the phenotype of BDNF mutants, although it did enhance alcohol consumption in the wild-type, indicating that the effects of BDNF on alcohol consumption is mediated through TrkB signaling (Jeanblanc et al., 2009; Hensler et al., 2003). Another study found that both acute and self-ethanol administration enhances dorsal striatal BDNF expression, while increased BDNF expression reduced ethanol consumption (McGough et al., 2004).

Only recently neurotrophic factors were identified in *Drosophila.* *Drosophila* Neurotrophin 1 and 2 (DNT1 and DNT2) (Zhu et al., 2008) have the Cystein-knot motif of mammalian neurotrophins. DNT1 appears to have neurotrophin-related functions, since DNT1 mutants show increased neuronal death and DNT1 expression reverses the phenotype (Zhu et al., 2008). It is unclear whether DNT1 mutants have altered ethanol sensitivity, although given its role in mammals, it can be hypothesized that *Drosophila* neurotrophins will function in a similar fashion. Particularly, because the pars intercerebralis region in flies, which contains neurons that synthesize neurosecretory peptides such as the insulin-like *Drosophila* peptide Dilp2 (Corl et al., 2005), has been shown to have pivotal roles in ethanol-sensitivity behavior (Corl et al., 2005), although it remains to be seen whether or not DNTs are expressed in this region.

Insulin Receptor Signaling Pathway

InR signaling occurs by complex intracellular mechanisms that begin when secreted insulin peptides bind its receptor at the cell's surface of a postsynaptic cell. The *Drosophila InR* is highly conserved and can be activated through binding bovine or porcine insulin at a k_D =10 nM (Fernandez-Almonacid and Rosen, 1987). Immediately following insulin-*InR* binding, cis-trans activation and autophosphorylation of key sites in the alpha-to-beta segments of *InR* (Haring, 1989), creates docking sites where adaptor molecules recruit other proteins that synthesize phosphoinositides to enhance a cascade of signaling.

PI3K catalyzes phosphatidyl inositol-4, 5-bisphosphate (PIP_2) into phosphatidyl inositol-3.4.5-triphosphate (PIP_3) (Ruderman et al., 1990), which activates *PDK1*, mechanism required to phosphorylate and activate Protein Kinase B (PKB)/*AKT* (Vanhaesebroeck and Alessi, 2000). Once activated, *AKT* phosphorylation causes Forkhead Box protein O (*FOXO)* to be degraded (Plas et al., 2003) and it can also phosphorylate and inhibit Tuberous Sclerosis Complex 2 (TSC2) (Inoki et al., 2002). This phosphorylation destabilizes the TSC complex leading to inhibition of Rheb and activation of TOR, which activates of *S6K1* and S6K2 in mammals, and a single *S6* kinase in *Drosophila* (Ruvinsky et al., 2005; Saucedo et al., 2003). Activated S6K phosphorylates the ribosomal s6 protein, a component of the 40S ribosomal subunit, which is required for protein synthesis initiation (Ruvinsky et al., 2005).

InR signaling is essential to many physiological and cognitive processes, and it is not surprising that various diseases ranging from cancer and neurodegeneration to diabetes II can be originated by *InR* signaling disruptions (Mauro et al., 2001; Plum et al., 2005) in a tissue specific fashion. Corl and colleagues (2005) demonstrated that *InR* signaling could also regulate ethanol-induced responses in the *Drosophila* NS. Whereas neuronal reduction in *InR* signaling confers sensitivity to ethanol sedation, its activation results in resistance (Corl et al., 2005). Furthermore, the authors validated some activating and inhibiting steps along the pathway with respective ethanol sedation

phenotypes produced by manipulating *InR* signaling components in the nervous system. For instance, increased *InR* signaling produces resistance to ethanol sedation, and as expected, pan-neuronal expression of *FOXO* conferred sensitivity.

A follow-up paper by Eddison et al. (2011) provided discrepant evidence from that of Corl et al. (2005) with respect to the role of *InR* signaling in ethanol-induced responses. Eddison et al. (2011) showed that increasing *InR* signaling via CNS expression of *PI3K*'s catalytic subunit p110, *PDK1*, or *AKT* produced sensitivity to ethanol sedation, while genetic manipulations that decrease *InR* signaling such as CNS expression of PTEN, which inhibits *PI3K*'s mediated conversion from PIP_2 to PIP_3, or expression of *PI3K* dominant negative, and pan-neuronal knockdown of *AKT* conferred resistance to LOR. In addition, Eddison and colleagues (2011) show that NS expression of the TOR effector Rheb, produces ethanol sedation sensitivity, and propose a model by which TOR signaling mediates both behavioral ethanol responses and synapse number via a distinct pathway than PI3K/AKT (Teleman, 2010). One possibility for discrepant results with the report by Corl and colleagues (2005) might be that developmental transgene expression of *InR* signaling components by the strong driver Elav may have been produced compensatory mechanisms capable of modifying the phenotype.

Aside of its role in adulthood, *InR* signaling is also important for normal development and growth (Fernandez et al., 1995). The devastating effects of ethanol on fetal development are well documented (Barnes et al., 1981; Jones et al., 1976; Hanson et al., 1976). McClure et al (2009) demonstrated that *Drosophila* is an adequate model system to study fetal alcohol syndrome (FAS). In their study, developmental defects in ethanol-reared flies were dramatic. Growth and viability were severely compromised and was reflected by drastic decreases in neural progenitor proliferation, dilp2 expression and *InR* transcription levels. Ethanol-reared flies displayed alterations in ethanol behaviors. These impairments included increased hyperactivity to moderate ethanol doses, resistance to ethanol sedation, and failure to acquire rapid tolerance, with the last two behavioral phenotypes being rescued by expressing dilp2 in insulin producing cells (IPCs).

Other Substrates of Behavioral Ethanol Responses: Cellular Architecture

Mechanisms of cellular architecture have recently been demonstrated to be important for behavioral ethanol responses. A *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 et al., 2009). Indeed, actin regulation influences the level of acute ethanol responses in both mice and flies with mutations in the actin-capping regulator EPS8 (Offenhauser et al.,

2006; Eddison et al., 2011). Ethanol-induced actin depolymerization is reduced in cerebellar granule neurons (CGNs) cultured from EPS8 KO mice compared to control cultures. EPS8 KO mice, which are resistant to intoxicating ethanol effects also drink more alcohol in a two bottle choice paradigm (Offenhauser et al., 2006). Conversely, *white rabbit* (*whir*) mutants of the *RhoGAP18B* protein, which encodes a GAP domain for Cdc42 and *Rac1* GTPases, display strong resistance to ethanol sedation and reduced ethanol preference in a two-bottle choice paradigm (Rothenfluh et al., 2006; Devineni et al., 2009).

Synapse Number

The *Drosophila arouser* (*aru*) encodes a mutation in the gene EPS8 (Eddison et al., 2011), although both *Drosophila aru* and Eps8L homologues lack the actin-capping domains of the mammalian EPS8 (Eddison et al., 2011; Offenhauser et al., 2006). Whereas mutants of *aru* display increased synapse number, they are also sensitive to ethanol-induced sedation. Environmental manipulations like social isolation cause synapse number to be significantly reduced (Donlea et al., 2011). A reduction in synapse number induced by social isolation produces resistance to ethanol sedation. When *aru* mutants are socially isolated, their synapse number and ethanol response phenotypes are restored to normal levels (Eddison et al., 2001). Although the exact mechanism by which *aru* mediates synapse number remains unknown, the evidence provided for the role

of synapse number in *Drosophila* ethanol responses is solid, though verification in mammalian systems is still warranted.

Genetic manipulations affecting acute and tolerance responses to ethanol, such as *PI3K* or *Ras homolog enriched in brain* (*Rheb*) over-expression, or mutations in *hang* (reviewed above) have been shown to affect synapse number in the neuromuscular junction (NMJ) in the developing (Schwenkert et al., 2008) and the adult fly brain (Eddison et al., 2011). Alterations in synapse excitability/inhibition may be at the heart of the effects of synapse number in ethanol response phenotypes. It may well be that dysregulation of synaptic plasticity occurs as a result of major synapse number losses/gains (Bourne et al., 2011), thus differentially impinging on the nervous system's ability to cope in response to continued ethanol influx.

Pre and post-synaptic transmission mechanisms are important factors affecting a fly's tolerance to ethanol. A deficit in either *Syntaxin 1A* (*Syx1A*) or *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). Mutations in *Synapsin* (*syn*), which encodes a pre-synaptic vesicle scaffolding protein, produce hyper-tolerance to ethanol (Godenschwege et al., 2004).

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 NT release, defects in neurite growth, synaptic formation and/or maturation, or due to the inability to prioritize the assembly of ready-to-release vesicles from those in the reserve pool (Cesca et al., 2010).

Results and Methods

Arfip and Arf6 Suppress RhoGAP18B's Behavioral Resistance to Ethanol Sedation

To uncover genes that interact with the *Drosophila* Rho-GTPase activating protein *RhoGAP18B*, a screen was performed using 300 randomly selected P-element mutants on the third chromosome. Mutations were isolated that modified the semi-lethality phenotype of the strong *whir*^{3} allele, and subsequently verified whether these mutations could also modify the ethanol sedation resistance of *RhoGAP18B* mutants.

I identified CG1784, which encodes the only *Drosophila* homologue for mammalian *Arfaptins* (*Arfips*) as a suppressor of *whir*^{3}. *Arfips* were originally isolated as *Rac* binding partner and since *RhoGAP18B* activates *Rac* in vitro, while *Rac1* mediates *whir's* mutant ethanol effects *in vivo*, I was encouraged to study to *Arfip* in terms of behavioral ethanol sedation. Loss-of-function imprecise excisions mutants were generated, and I tested one of these for behavioral ethanol phenotypes (Fig S2). To validate the relevance of the performed screen to ethanol- induced responses, I tested whether a single copy of *Arfip* and *Arf6*, which have no phenotype compared to controls, would genetically interact with *whir* and modify their mutant ethanol resistance phenotype. Indeed, heterozygous mutations in *Arfip* and *Arf6*, which alone have no phenotye

compared to wildtype, partially suppressed the strong ethanol resistance *whir*^{1} mutants (Fig 1a and b).

The fact that a copy of either *Arfip* or *Arf6* suppressed *RhoGAP18's* mutant phenotype, it suggested that both *Arf6* and *Arfip* function in acute ethanol sedation in the opposite direction a *RhoGAP18B*. That is, while *RhoGAP18B* mediates ethanol sensitivity, *Arfip* and *Arf6* would mediate ethanol resistance. I then tested whether homozygous *Arfip* and *Arf6* mutants showed behavioral sensitivity to the sedative effects of alcohol. Indeed, *Arfip* and *Arf6* mutants showed strong sensitivity to alcohol sedation compared to controls (Fig 1c and d).

To determine whether Arf6 mutants absorbed and metabolized ethanol normally, I determined the internal ethanol concentration of flies after various exposure doses. Results show that although *Arf6* mutants require very low concentrations of ethanol to arrive at the ST-50, they absorb ethanol at the same rate as their WT counterparts in all time points assessed (Fig S2), indicating that the behavioral phenotype in Arf6 mutants is caused by a pharmacodynamic, and not pharmacokinetic defect.

Arfip and Arf6 Regulate Behavioral Ethanol-Resistance in the Same Genetic Pathway as RhoGAP18B

Biochemical studies have previously shown that *Arfip* and *Arf6* function synergistically to mediate actin dynamics in various cell lines (D'Souza-Schorey et al., 1997). To test whether *Arfip* and *Arf6* function in the same pathway in vivo, I generated *Arfip/Arf6* double mutants and exposed them to sedating levels of ethanol vapors**.** I hypothesized that if *Arf6* and *Arfip* function in a linear pathway, the loss of *Arfip* would not exacerbate the ethanol sensitivity phenotype of *Arf6* mutants. The *Arf6/Arfip* double mutants are equally sensitive as *Arf6* mutants (Fig 1e and f). To exclude the possibility of a 'floor effect', i.e. *Arf6* mutants had already reached maximum possible ethanol sensitivity; I performed another experiment under two altered conditions. First, I lowered the ethanol/air mixture to delay sedation, and achieve a wider dynamic range. Second, I tested the ethanol sensitivity of *Arf6* double mutant together with the *cheapdate* mutant. *Cheapdate (Chpd)* is an ethanol sensitive mutant of the *Amn* gene, which functions in the PKA pathway and is not hypothesized to function in the same genetic pathway as *Arf6* (Fig 1F).

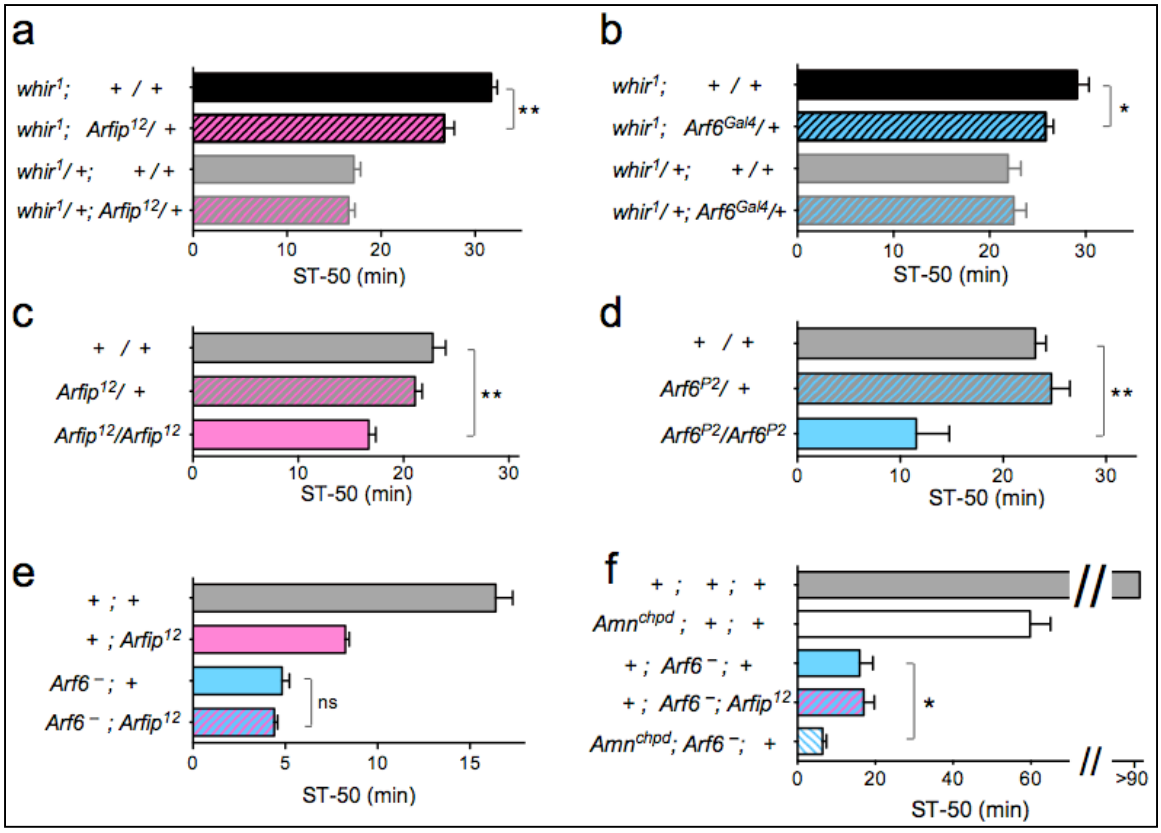


Figure 1: Arfip and Arf6 Regulate Behavioral Ethanol-Resistance in the Same Genetic Pathway as RhoGAP18B. In this, and the following graphs, bars represent means ±SEM. Unless otherwise noted, flies were exposed to (130/20) ethanol/air-flow rate, and the median sedation time (ST-50) was determined as described11. (a,b) Heterozygous Arfip or Arf6 mutations partially suppress the ethanol-resistance phenotype of whir1 (** P =0.003, n = 6, a, and * P = 0.049, n = 8-10), while not altering the wild-type phenotype of whir1 /+. (c,d) Homozygous Arfip or Arf6 mutations are sensitive to ethanol-induced sedation (** P < 0.001, n = 4-9). Note that all Arf6 alleles utilized, Arf6P2, Arf6KG, and Arf6Gal4 showed the same sensitivity phenotype (data not shown; also see Fig. 2b). Also, the Arf6 phenotype was not caused by pharmacokinetic alterations (see Supplementary Fig. 2). (e,f) Arfip mutation does not increase the sensitivity of Arf6– mutant flies (e, Arf6P2/Gal4, ns = not significant, P = 0.30, n = 9-14 per genotype), while the ethanol-sensitive cheapdate allele of the Amnesiac gene further increased Arf6– ethanol-sensitivity (f, * P = 0.036, n = 5 per genotype). Note that flies in (f) were exposed to a very low ethanol flow-rate of 20/130 ethanol/air, to increase the resolution of the very sensitive phenotypes. At this low concentration, wild-type flies did not sedate within 90 minutes of exposure (Peru y Colón de Portugal et al. Unpublished)

I posited that if *Arf6* and *Arfip* function in the same pathway, only the *Arf6/Chpd* double mutant would show increased sensitivity compared to *Arf6/Arfip* and *Arf6* mutants. While the double mutants *Arfip/Arf6* show no

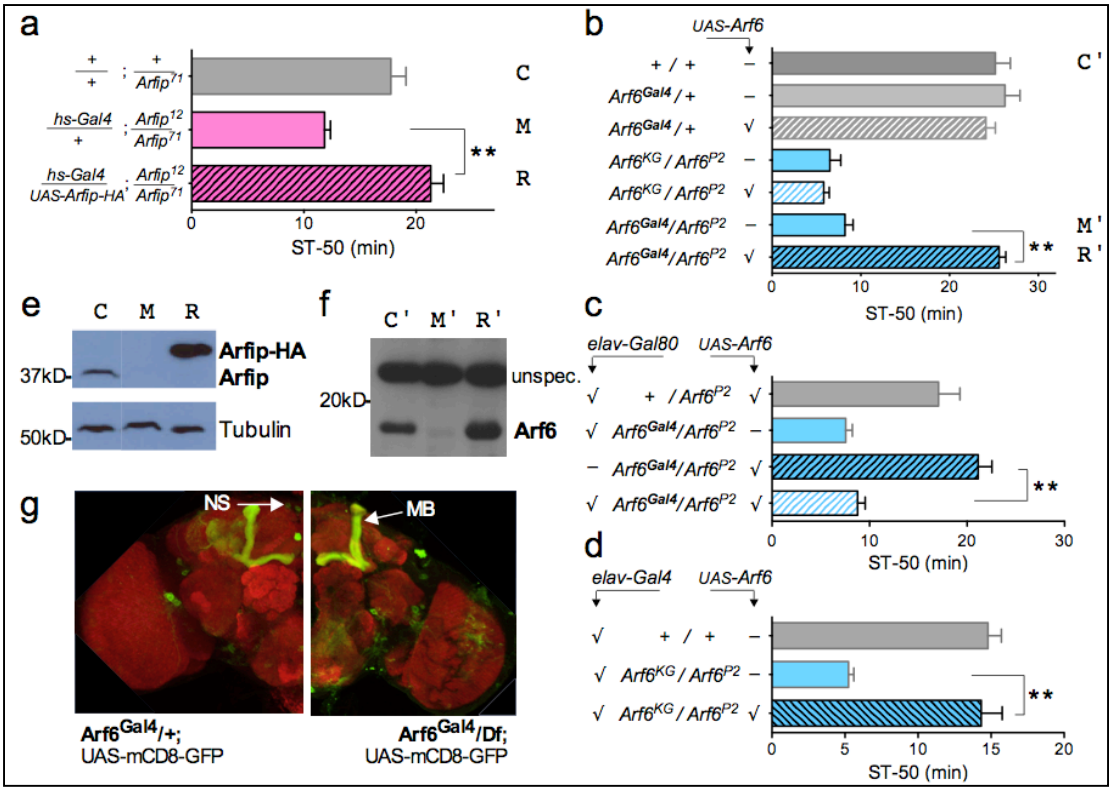
difference compared to *Arf6* mutants, *Arf6/Chpd* double mutants exhibit increased sensitivity to ethanol sedation compared to *Arfip/Arf6* mutants consistent with *Arfip* and *Chpd* functioning in distinct pathways.

Arf6 is Necessary and Sufficient in the NS for Proper Behavioral Responses to Ethanol

To establish whether the behavioral sensitivity to the intoxicating effects of ethanol displayed by $Arfip^{x12}$ (Fig S2b) is produced by the lack of *Arfip*, the cDNA of *Arfip* was reinserted in the *Arfip* mutant employing a *Heat Shock* Gal4 driver (*hs-Gal4*) at the permissive temperature of 29C using the UAS/Gal4 binary, and this genetic manipulation produced resistance to ethanol sedation in the Arfip mutant, thus rescuing the mutant ethanol sensitivity (Fig 2a). To ask whether increased ethanol sensitivity in *Arf6* mutants is caused by the loss of *Arf6*, we performed an *Arf6* rescue experiment, where a Gal4 expressing *Arf6* allele ($Arf6^{Gal4}$) was used to drive the expression of UAS *Arf6* cDNA into the *Arf6* mutants. This UAS *Arf6* transgene was genetically recombined together with the $Arf6^{P2}$ mutant allele.

When $Arf6^{P2/Gal4}$ transheterozygous mutants carrying the UAS-*Arf6* transgene are tested for their response to ethanol sedation, these flies display normal ethanol sedation sensitivity, indicating a complete rescue of the mutant phenotype. Importantly, introduction of the UAS-*Arf6* transgene into the $Arf6^{P2/KG}$

transheterozygous mutant in which GAL4 is not expressed, failed to rescue the



igure 2: Arf6 is Necessary and Sufficient in the CNS for Proper Behavioral Responses to Ethanol. (a) Expression of an Arfip transgene under control of the Gal4 DNA-binding domain UAS (UAS-Arfip-HA) with the ubiquitously expressed hs-Gal4 driver rescues Arfip12/71 ethanol-sensitivity (** $P < 0.001$, n = 6-10 per genotype). Flies were reared and kept at 29°C, sufficient for leaky hs-Gal4 and transgene expression, see (e). (b) Gal4-expression from the Arf6Gal4 mutant allele rescues the Arf6 sensitivity phenotype when a UAS-Arf6 transgene is introduced (** $P < 0.001$, n = 6-7). In this, and following panels, checkmarks indicate the presence of the driver, or transgene above/below. (c) Gal4 nervous system expression from Arf6Gal4 is necessary for rescue, since neuronal expression of the Gal4-inhibitor, Gal80 (elav-Gal80), suppresses rescue (** $P < 0.001$, n = 7-8). (d) Neuronal expression of UAS-Arf6 (with the weak elav3E1-Gal4 driver) is sufficient to rescue the Arf6 mutant ethanol-sensitivity (** $P < 0.001$, n = 5-6). (e,f) Restoration of Arfip (e), and Arf6 (f) protein signal on western blots made with extracts from behaviorally rescued flies (see a and b respectively for genotypes, and Supplementary Fig. 1 for additional loss-of-function characterization). (g) Expression of Gal4 from the Arf6Gal4 allele in wild-type Arf6 Gal4/+(left) and Arf6Gal4/Df mutant brain (right). Regions stained include the mushroom bodies (MB) and neurosecretory cells (NS), which are known to be involved in behavioral ethanol responses. No gross morphological aberrations are obvious in the mutant (see text) (Peru y Colón de Portugal et al. Unpublished).

strong sensitivity to ethanol sedation mutant phenotype. The experiment confirms that the absence of *Arf6* expression is solely responsible for the increased behavioral sensitivity to ethanol intoxication in the *Arf6* mutants.

When examining the neural expression of the *Arf6*^{Gal4} driver with cytoplasmic GFP (Fig 2g), we see that it drives in restricted brain regions in the adult fly, inducing in the mushroom bodies. This data suggests that *Arf6* expression for ethanol responses may be restricted to the nervous system. To determine whether *Arf6* is necessary in neurons to regulate ethanol's sedating effects, the *Arf6* cDNA transgene was driven using *Arf6*^{G4}. However, *Arf6*^{G4} driven expression in the NS was selectively blocked by addition of *Elav-Gal80* (Fig 2c). NS expression blockade resulted in sensitivity to ethanol sedation in the mutant, whereas in the absence of expression blockade, addition of the *Arf6 cDNA* transgene in the mutant resulted in complete phenotypic restoration

I next sought to establish whether Arf6 expression specifically in the nervous system (NS) was sufficient to rescue ethanol intoxication. To this end, we expressed an *Arf6 cDNA* transgene in neurons using the nervous system driver *elav-Gal4*^{3E1} in the *Arf6* mutant. Nervous system expression of Arf6 completely restored the sedation sensitivity of *Arf6*$^{KG/P2}$ (Fig 2d).

Arf6 is Necessary and Sufficient in the Adult, but not During Development to Control Behavioral Sensitivity to Ethanol Sedation

Arf6 has been previously implicated in neurodevelopmental processes, including neurite growth and development of dendritic spines (Hernández-Deviez et al., 2002; Choi et al., 2006). *Arf6* might be required in a sedation-circuitry, or

acutely in the adult CNS. To elucidate the temporal requirements of *Arf6* in the regulation of acute ethanol intoxication in flies, two Arf6 rescue experiments were performed.

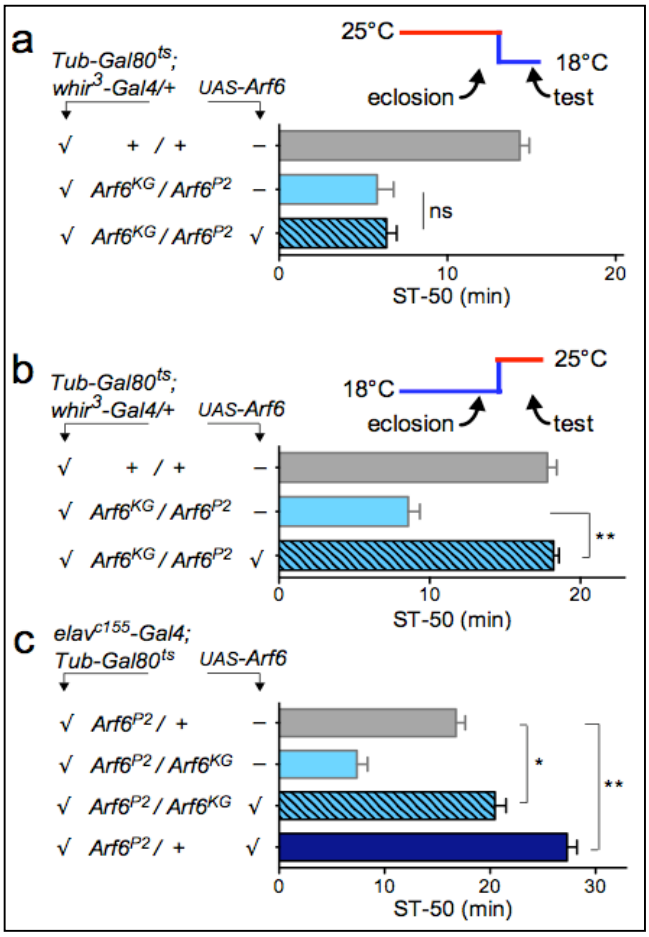


Figure 3: Arf6 is Necessary and Sufficient in the Adult, but not During Development to Control Behavioral Sensitivity to Ethanol Sedation. UAS-Arf6 expression was suppressed utilizing Gal80ts, which inhibits Gal4 at 18°C but not 25°C. The flies were shifted to the experimental temperature for three days after eclosion (see small schematics). (a) Expression of UAS-Arf6 during development, utilizing whir3-Gal4/+ as a driver, does not rescue Arf6 ethanol sensitivity (ns, P = 0.63, n = 11 per genotype). (b) UAS-Arf6 expression in the adult only rescues the Arf6 ethanol-induced sensitivity (**P < 0.001, n = 8). (c) Strong nervous system expression of UAS-Arf6 (with the elavc155-Gal4 driver) causes lethality. When expressed in the adult only (same temperature regimen as in b), it rescues mutant Arf6 beyond wild type (stippled bar, * P < 0.05), and causes strong ethanol-resistance in wild type (dark blue bar, ** P < 0.01, Tukey's multiple comparison test, n > 7) Peru y Colón de Portugal et al. Unpublished

To address whether *Arf6* is required selectively during development, a UAS *Arf6* cDNA transgene was driven during development but not in the adult using the GAL4-encoding *RhoGAP18B* allele, *whir*^{3}. Two copies of Tubulin-Gal80^{ts} were employed to suppress GAL4-driven expression at the restrictive

temperature of 17°C. In this first experiment, flies were raised at 25C, transferred for 3 days to 17°C and tested. To investigate a requirement of *Arf6* in the adult fly, the opposite temperature conditions were performed. That is, flies were raised at 17°C and subsequently transferred to 25°C for 3 days prior to testing. Whereas *Arf6* expression in *Arf6* mutants transferred from 25°C to 17°C resulted in increased sensitivity to the sedating effects of ethanol, *Arf6* expressing mutants transferred from 17°C to 25°C exhibited complete restoration of the mutant phenotype, demonstrating that *Arf6* is sufficient and necessary in the adult fly to regulate ethanol-induced responses. Therefore, there is no developmental requirement for Arf6 function.

An Arf6 rescue experiment was performed using the stronger neural driver $Elav^{C155}$ to ask whether Arf6 expression in the NS is sufficient to rescue ethanol-induced responses (Fig 3C). Developmental expression of Arf6 using this driver caused lethality in larvae, therefore a single copy of temperature sensitive *Tubulin-Gal80^{ts}* was used to selectively enable adult expression of Arf6. While a single copy of *Tubulin-Gal80^{ts}* suppressed larvae lethality, partial ethanol response rescue was obtained in flies kept at a restrictive temperature of 20C throughout development and in the adult, indicating that one copy of *Tubulin-Gal80^{ts}* was not sufficient to fully suppress Gal4 driven expression in the adult

(data not shown). However, when adult flies were transferred to 20C to 29C for 3 days, a resistance phenotype was produced in the rescue condition (Fig 3C).

Arfip Integrates the Rho-Family and Arf6 Signaling Pathways through Binding to GTP Forms of Rac1 and Arf6 GTPases

While several studies have indicated that *Arfip* exclusively binds to the GTP bound form of *Arf6* (Tarricone et al., 2006; Shin et al., 2001). Other studies have shown that *Arfip* also binds to GTP or GDP forms of *Rac1* (Shin et al., 2001; Tarricone et al., 2001). Therefore, the question of whether *Arfip* binds to either GTP/GDP forms *Rac1* and *Arf6* in Drosophila S2 cells was investigated (Fig 5a and b). A FLAG-Arfip expressing cell line was generated and transiently transfected with either GTP/GDP forms of *Rac1* and *Arf6*, which were tagged with yellow florescent protein.

Figure 4a shows that *Arfip* preferentially interacts with *Rac1*.*GTP*-locked mutant, although it can still bind to *Rac1-GDP* (Fig. 4a). As with mammalian Arfaptin 2, Immunoprecipitation experiments show that *Drosophila Arfip* binds preferentially to *Arf6 GTP* over its inactive *Arf6 GDP* nucleotide (Fig 4b). Therefore, Arfip can bind to activated Arf6 and Rac (Tarricone et al., 2001), and together regulate ethanol sedation behavior (Rothenfluh et al., 2006).

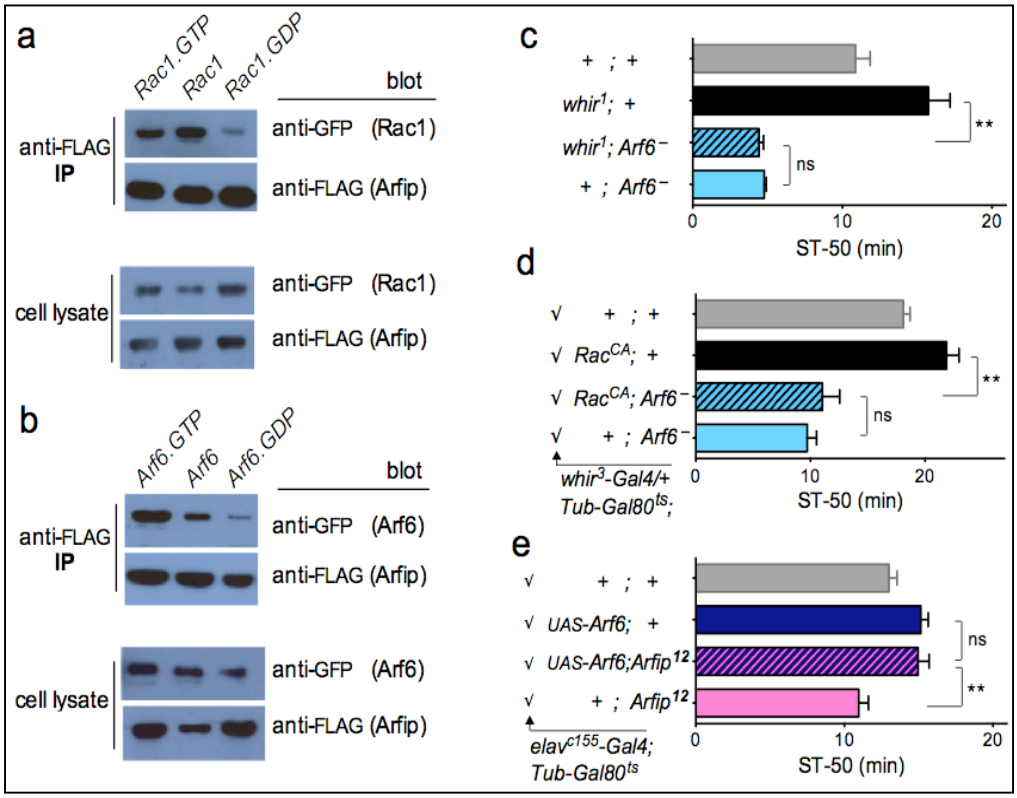


Figure 4: Arfip Integrates the Rho-to-Arf Pathway through binding to GTP forms of Rac1 and Arf6 GTPases. (a,b) Arfip preferentially binds to the GTP-locked forms of Rac1 (a) and Arf6 (b). Drosophila S2 cells, stably expressing Arfip-FLAG, were transiently transfected with Rac1-YFP, or Arf6-YFP. GTPase pulled down with anti-FLAG beads was then detected with anti-GFP antibody, and representative pull-downs of multiple independent ones are shown. Mutants used were Rac1G12V (GTP-locked), Rac1T19N (GDP-locked), Arf6-Q67L (GTP-locked), and Arf6T44N (GDP-locked). (c,d) Arf6– double mutants with ethanol-resistant whir1 (c) or activated Rac1 (UAS-Rac1^{CA}), driven with whir3-Gal4/+ in the adult only using Tub-Gal80ts to avoid developmental lethality (see Fig. 3b) are no different from ethanol-sensitive Arf6 mutants alone, indicating that Arf6 acts downstream of RhoGAP18B and Rac1. (ns = not significant, P = 0.25, ** P < 0.001, n > 7 per genotype, c, and ns = not significant, P = 0.44, ** P < 0.001, n > 6 per genotype for d). Arf6– is Arf6KG/P2. (e) The ethanol-resistant UAS-Arf6 overexpression phenotype is unchanged when ethanol-sensitive Arfip12 is introduced, indicating that Arf6 acts downstream of Arfip. (ns = not significant, P = 0.85, ** P < 0.001, n > 6 per genotype. Pan-neuronal elavc155-Gal4/+ was used to drive UAS-Arf6 in the adult as in Fig. 3c)(Peru y Colón de Portugal et al. Unpublished).

Arf6 Mediates Ethanol Sedation Downstream of Arfip and Rac1

To investigate how signal transduction between Arf6, Rac1, and Arfip is hierarchically conveyed, I performed a genetic epistasis experiment, which compared the ethanol response sensitivity of *Arf6* and *RhoGAP18B* mutants to a double *Arf6* and *whir*^{1} double mutant. I initially reasoned that if *Arf6* functioned

downstream of *RhoGAP18B*, the phenotype in this double mutant would phenocopy the *Arf6* mutant and not the *whir*^{1} ethanol resistance mutant (Fig 4c). While the *whir* mutant shows ethanol resiance, the *whir1/Arf6* double mutant shows ethanol sensitivity, suggesting that *Arf6* functions downstream of *RhoGAP18B*.

Many studies using various cell culture systems have shown that *Arf6* and *Rac1* function in the same pathway, although with conflicting results as to the epistatic relationship between *Rac1* and *Arf6*. To this end, I expressed constitutive active *Rac1* (*G12V*) in the *Arf6* mutant background using *whir*^{3}*-Gal4* as driver. Note, that as heterozygotes, whir3/+ displays wild-type ethanol sensitivity (Rothenfluh et al., 2006). To circumvent developmental lethality caused by *Rac1*^{CA} expression, *Tubulin-Gal80*^{ts} was used to control the temporal expression of *Rac1* via temperature. While expression of *Rac1*^{CA} in the wild-type background results in resistance to ethanol sedation, expressing *Rac1*^{CA} in the *Arf6* mutant background produces sensitivity to ethanol-induced sedation indistinguishable from *Arf6* ethanol-sensitivity. This data indicates that Arf6 functions downstream of *Rac1* and *RhoGAP18B*.

Expression of Arf6 in the wild-type nervous system produced strong ethanol resistance, which allowed us to perform an experiment where *Arf6* would be expressed in *Arfip* mutants to ask whether *Arfip* would be epistatic over *Arf6*. Because our IP experiments support the hypothesis that *Arfip* is a mediator of a

cross talk between *Rac1* and Arf6, and my genetic epistasis experiments show that *Arf6* functions downstream of *Rac1*, we hypothesized that *Arf6* would regulate ethanol sedation downstream of *Arfip* (Fig 4e). To test our hypothesis, I expressed *Arf6* in the nervous system of *Arfip* mutants and WT using the pan-neuronal driver *elav^{C155} –Gal4,* including one copy of *Tubulin-Gal80^{ts}* to prevent developmental lethality. Contrasting the behavioral sensitivity of *Arfip* mutants, *Arf6* expression in the NS of *Arfip* mutants conferred behavioral resistance to ethanol sedation, which was no different than in the WT, indicating that *Arf6* controls behavioral responses downstream of *Arfip*.

Arf6 Regulates Insulin- Induced S6K-P Upstream of PI3K

Recent evidence suggests that *Arf6* is required for insulin-induced *AKT* phosphorylation (*AKT-P*) in HeLa cells, suggesting that *Arf6* is an upstream regulator of *AKT* (Lim et al., 2010). Other reports have examined whether *Arf6* has a role in *InR* signaling but did not find a requirement for *Arf6* (Yang and Mueckler, 1999; Lawrence and Birnbaum, 2001; Bose et al., 2001). *AKT* regulates *S6K* by directly binding and phosphorylating *Tuberous Sclerosis Complex 2* (*TSC2*). This phosphorylation destabilizes the *TSC* complex leading to inhibition of *Rheb* and activation of *TOR*, which in turn phosphorylates and activates of *S6K*, while inhibiting translational inhibitor and *AKT* effector, *4E-BP*. Because *Arf6* was shown to function upstream of *AKT* and insulin-induced

activation of *AKT* leads to downstream of activation of *S6K*, I thus posited that *Arf6* was required for *S6K-P.*

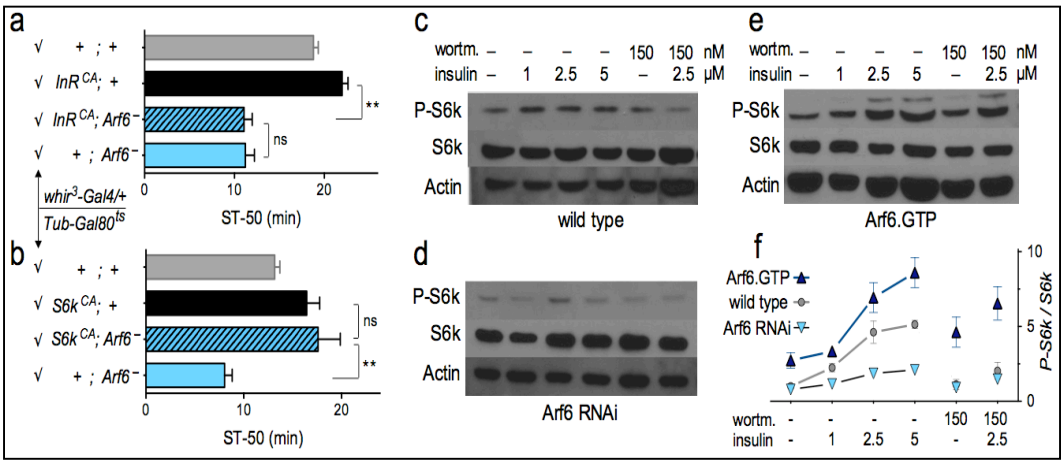


Figure 5: Arf6 Positively Regulates Insulin- Induced Activation of S6K to Control Behavioral Ethanol Responses
a) Arf6– double mutants with ethanol-resistant activated insulin receptor (UAS-InRCA) are as sensitive as Arf6– mutants alone, indicating that Arf6 acts downstream of InR. (ns = not significant, P = 0.92, ** P < 0.001, n > 6 per genotype). (b) Arf6– double mutants with ethanol-resistant activated S6 kinase (UAS-S6kCA) are as resistant as S6kCA mutants, indicating that S6k acts downstream of Arf6 (ns = not significant, P = 0.64, ** P < 0.001, n > 5 per genotype). Both UAS-InRCA and UAS-S6kCA were expressed using whir3-Gal4/+ in the adult only using Tub-Gal80ts to prevent developmental lethality. Arf6–is Arf6KG/P2 in (a) and (b). Also see Supplementary Fig. 3 for adult neuronal phenotypes obtained with perturbations in InR signaling. (c-f) Representative western blots with quantitation (f, n=3) showing an increase of S6k phosphorylation (P-S6k) in serum-starved Drosophila S2 cells (c) exposed to insulin. The increase of S6k-P is blocked by the Pi3 kinase inhibitor wortmannin. P-S6k increase by insulin is strongly reduced in Arf6 RNAi-depleted cells (d), and augmented in activated Arf6Q67L- overexpressing cells (e). Note that 200nM wortmannin efficiently inhibits P-S6k in Arf6 Q67L-overexpressing cells (data not shown) (Peru y Colón de Portugal et al. Unpublished).

Acute Ethanol Intoxication and Lack of Arf6, Both Cause Loss of S6K-P

Corl and colleagues (2004) demonstrated that in *Drosophila*, neuronal In*R* signaling reduction confers ethanol-sensitivity, while iInR increase produces resistance to sedating ethanol effects. It remained to be confirmed whether *Arf6* could regulate *InR* signaling in *Drosophila* and if so, whether *Arf6* mediated control of *InR* signaling regulates ethanol-induced responses. To ask whether *Arf6* mediates InR signaling, genetic epistasis experiments were performed between *Arf6* and *InR* using *whir*^{3} driver together with a copy of *Tubulin-Gal80*^{ts} to

avoid compensatory-response phenotypes associated with developmental transgene expression (Fig 5a). I found that expressing the *InR-*^{CA} in the WT produced strong resistance to ethanol sedation, while *InR-*^{CA} expression in the *Arf6* mutant resulted in strong sensitivity to ethanol intoxication indicating that *Arf6* functions in the insulin signaling pathway downstream of the *InR*.

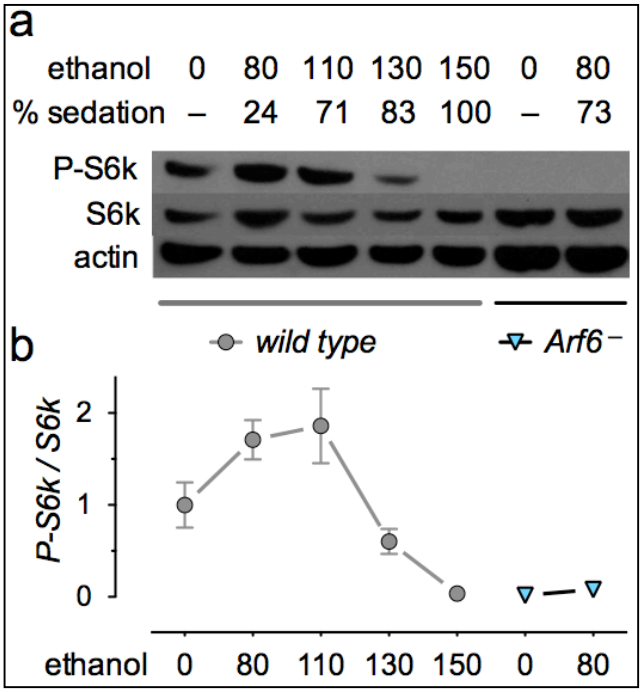


Figure 6 Acute Ethanol Exposure and Lack of Arf6, Both Cause Loss of S6K-P:(a) Western blot of wild-type Drosophila head extracts showing loss of S6 kinase phosphorylation (P-S6k) with increasing dose of ethanol exposure (left six lanes). In Arf6– mutant extracts, P-S6k is not detected, regardless of ethanol exposure (right two lanes). In this panel, the dose is indicated as ethanol flow rate, where ethanol/air flow rate = 150. Flies were exposed for 20 minutes, and the average percentage of flies sedated is indicated. Arf6– is Arf6KG/P2. (b) Quantitation of the data shown in (a), with n=3 independent blots. (c) Model of the Arf6-mediated signaling pathway to control ethanol sedation, see text. Cyth2 is Cytohesin 2, also known as ARNO. Note that ethanol inhibits S6k phosphorylation via an unknown, and not necessarily direct mechanism (Peru y Colón de Portugal et al. Unpublished)

In light of the evidence that increasing *InR* signaling produces resistance to ethanol sedation, and that InR is a positive regulator of AKT/TOR effctor S6K, we hypothesized that expression of *S6K*^{CA} would lead to ethanol-induced resistance to ethanol intoxication both in the WT. Because Arf6 functions downstream of InR, it was unclear whether Arf6 would function upstream or downstream of S6K in *Drosophila*. To test our hypothesis, I assayed the ethanol sensitivity of *S6K*^{CA} over expression in the WT and in the *Arf6* mutant using the

whir^{3} driver together with a copy of Tubulin-Gal80^{ts} (Fig 5b). I found that *S6K*^{CA} leads to resistance to ethanol sedation in both instances, indicating that *S6K* is a downstream mediator of *Arf6*'s resistance to ethanol sedation.

Next, I hypothesized that if *Arf6* controls *S6K* activation in response to insulin, then *Arf6* mutants would have reduced baseline *S6K-P* compared to controls. To test this hypothesis, western blot experiments were performed using protein extracts of WT and *Arf6* mutant heads. Compared to WT, *Arf6* mutants are completely deficient in baseline *S6K-P* (Fig6 a and b). These results demonstrate that *Arf6* is a key regulator of *InR* signaling *in vivo*. I next asked whether ethanol exposure and ethanol sedation affected *S6K*-P *in vivo* and if so, whether behavioral sedation.

Experiments in human subjects have found that low ethanol levels stimulate *InR* signaling, while high levels impair it (Facchini et al., 1994; he et al., 2007). Thus, I hypothesized that ethanol sedation would alter *S6K*-P in a similar manner. That is, levels of *S6K*-P would be higher in flies exposed to ethanol conditions conferring minimal sedation, while *S6K*-P would be reduced in flies exposed to ethanol concentrations producing maximal sedation (Fig 6a and b). WT flies were exposed to ethanol for 20 minutes at different ethanol/air flowrates, and at the end of the 20-minute exposure, they were immediately frozen with dry ice, decapitated, and protein extracts were prepared from fly heads. *S6K-P* levels

were monitored in fly head protein extracts using anti-*S6K-P* antibody. Dosages of ethanol vapors used ranged from low to high (E/As: 80/70, 110/40, 130/20, 150/0).

Flies exposed to the lowest dosage (80/70) exhibited an increase in *S6K-P* and 24% sedation at the end of the 20 minute exposure. Moderate ethanol dosages (110/40 and 130/20) resulted in dose dependent *S6K-P* decrease with flies exhibiting increasing levels of sedation. The maximal dosage (150/0), which conferred 100% sedation, also resulted in the complete depletion of *S6K-P*. Indicating that ethanol-induced sedation is correlated with loss of *S6K-P*, suggesting baseline *S6K-P* levels may be indicative of an organisms' behavioral sensitivity to ethanol exposure. Taken together, results of these sets of behavioral and biochemical assays in cell culture and *in vivo* argue that ethanol sensitive Arf6 mutants are pre-sensitized to ethanol's sedating effects due to deficient baseline S6K-P. Thus, lack of S6K-P makes Arf6 mutants hypersensitive to ethanol, which sedate with less with than 17mM internal ethanol concentration (Fig S1), no wonder they are indeed more sensititive than the ethanol sensitive *Chpd* mutant, which sedates at 60 minutes compared to 20 minutes for the Arf6 mutant at doses that are innocuous to the wildtype (Fig 1f).

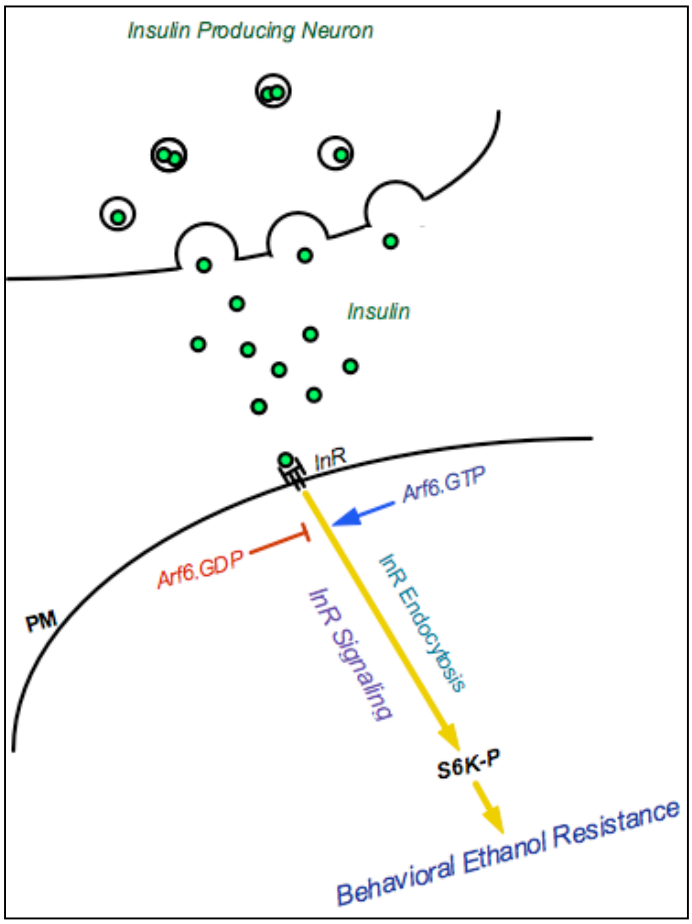


Figure 7: Arf6 Controls InR Signaling and Acute Ethanol Behaviors via Endocytosis and Recycling of InR: A Proposed Model. Arf6 GTP promotes InR internalization and insulin-induced S6K-P, which mediates behavioral resistance to ethanol sedation. Conversely, Arf6-GDP inhibits InR endoctytosis, and thus decreasing S6K-P, which results in behavioral sensitivity to ethanol sedation.

Supplemental Figure Legends

SUPPLEMENTAL INVENTORY

Figure S1 shows that *Arf6* mutants have no change in ethanol absorption and metabolism, thus supporting the primary phenotype (Figure 1) as Pharmacodynamic. Figure S2 shows the *Arf6* and *Arfip* gene structure, and indicates that mutations therein behave as amorphs, thus allowing rescue, as shown in Figure 2. Figure S3 shows phenotypes obtained by adult neuronal perturbation of the insulin pathway, thereby supporting Figure 5.

SUPPLEMENTAL DATA

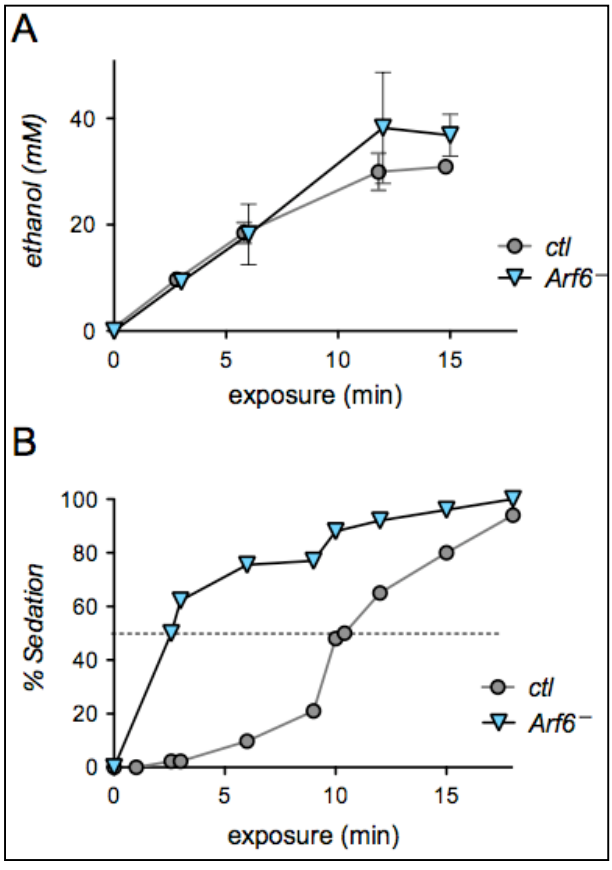


Figure S1: Unchanged Ethanol Absorption and Metabolism in Arf6 Mutant Flies. Flies were exposed to 150/0 ethanol/air, flash frozen and their internal ethanol concentration measured. 2-way ANOVA indicates significant ethanol increase over exposure time ($p < 0.001$, $n = 3$ per time and genotype), but no effect of genotype ($p > 0.74$). The Arf6 mutant genotype is Arf6P2/Gal4. B, Fraction of sedated flies from A. The stippled line indicates the ST-50 (Peru y Colón de Portugal et al. Unpublished).

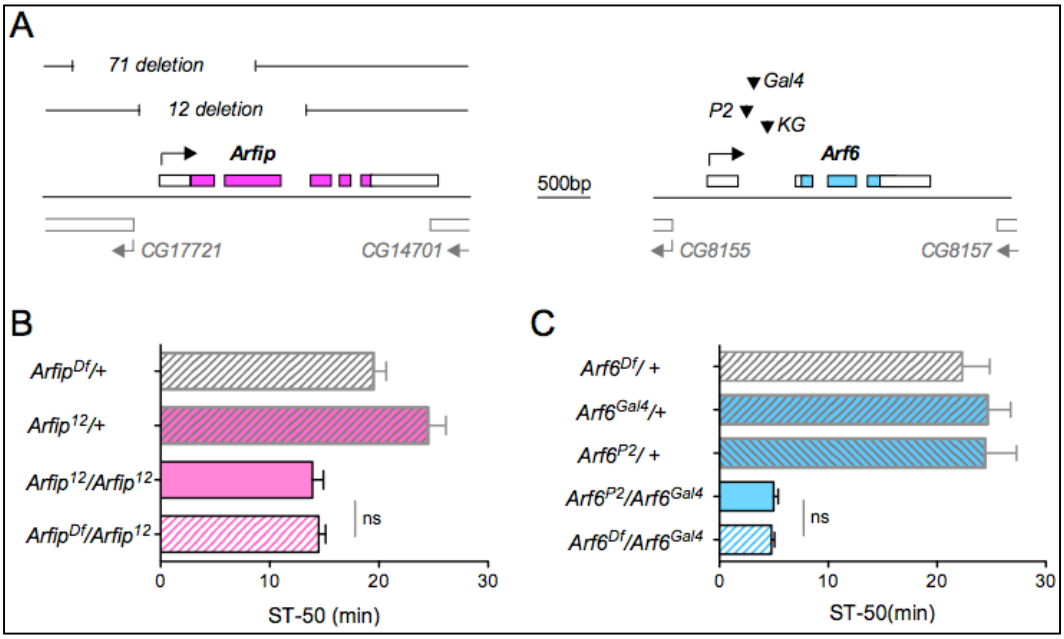


Figure S2: Schematic Representation of the Drosophila *Arfip* and *Arf6* Loci and Mutations, which Act as Amorphs (Enabling Rescue in Figure 2). (A) Exons are indicated by boxes, with the open reading frame in color. Small deletions of *Arfip*, induced by imprecise P-element excision are indicated above (left), and transposon insertions in the *Arf6* gene are indicated by triangles (right). (B, C) Large deficiencies completely removing the *Arf6* gene (B), or *Arfip* (C), were crossed to other alleles. The mutant transheterozygous Df genotype was no more severe that the other mutant phenotypes (n>7, ns p>0.62), indicating that the non-Df alleles are amorphic (null) (Peru y Colón de Portugal et al. Unpublished).

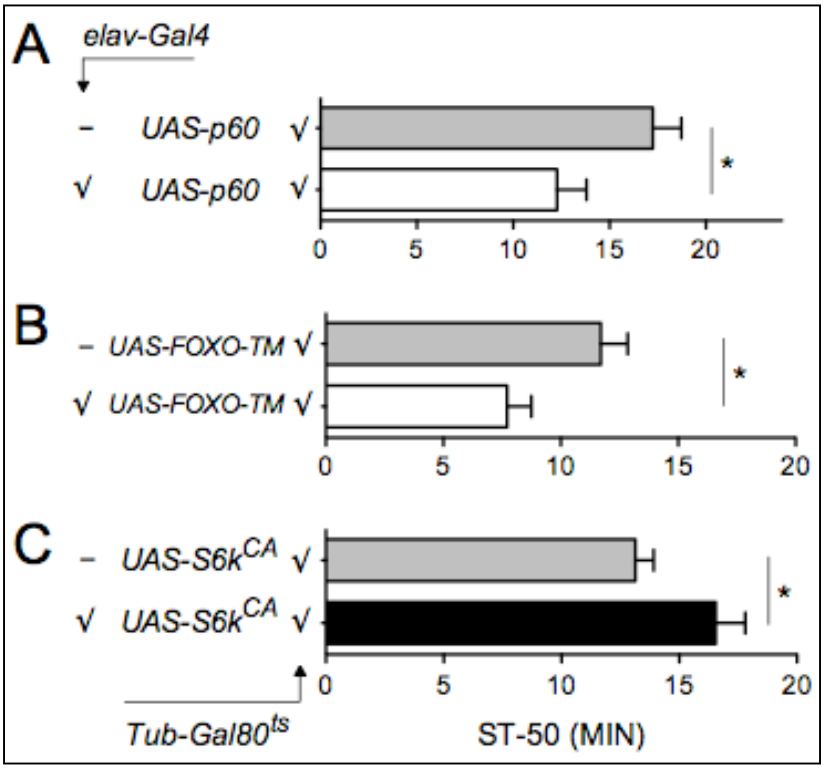


Figure S3: Adult Neuronal Changes in Insulin Receptor Signaling Cause Behavioral Ethanol Phenotypes, Supporting Figure 5. (A-C) Flies containing Tubulin-Gal80ts were raised at 17°C, then shifted to 29°C for 3 days during adulthood to allow UAS-transgene expression, and then tested for ethanol-induced sedation at E/A=130/20. Expression of the Pi3 kinase inhibitor p60 (A, Weinkove et al., 1999), or activated forkhead transcription factor *FOXO*-TM which is negatively regulated by insulin signaling (B, Junger et al., 2003) caused sensitivity, while activated *S6K* expression led to ethanol-resistance (C). n>5 per genotype, *p<0.05 ((Peru y Colón de Portugal et al. Unpublished).

EXPERIMENTALPROCEDURES

Fly Stocks, Genetics, and Behavioral Experiments

Flies were maintained on regular cornmeal/yeast/molasses at 25°C/65% humidity (unless otherwise specified). Flies were out-crossed for at least 5 generations to the *w1118* Berlin genetic background. The following fly strains were obtained from the Bloomington stock center: *Arf6P2* (EP2612, #17076), *Arf6KG* (KG02753, #13763), *Arfipd04253* (#19201, the original *whir3*-interaction strain). *Arfip12* was generated by imprecise excision of *ArfipUM-8176-3* (Szeged stock center). *Arf6Gal4* was obtained from the Kyoto stock center (NP5226, #104910). Ethanol exposure and determination of the ST-50 via measuring the flies' loss-of-righting reflex was performed as described (Rothenfluh et al., 2006).

Ethanol Sedation Assay

The ST-50 was used as a measure in all ethanol-induced sedation experiments and experiments were performed as described in (Rothenfluh et al., 2006). The ethanol/air pressure ratio varied depending on the purpose of the experiments (For more information see the result section).

Ethanol Absorption Assay

Ethanol concentration in flies was measured using the ethanol reagent kit (# 229-29) from *Genzyme Diagnostics*. Millimolar (mM) ethanol concentration in flies

was calculated in accordance to the protocol provided by the vendors. WT and *Arf6* mutants (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 **(S2)** and sedation was monitored through out the exposures. At the end of the exposures, flies were frozen in dry ice and homogenized. Tissue preparation as performed as described in (Moore et al., 1998).

Immunohistochemistry

An *Arf6*^{G4}/Cyo; UAS-mCD8GFP stock was produced and virgin flies were crossed to W. Berlin and *Arf6*^{DF} mutant males. F1 progeny absent of balancers were collected. Flies were decapitated and their brains dissected in either PBT or HL3 solutions. Immunostaining of fly brains was carried out as described in (Wu & Luo, 2006). GFP labeling was performed by incubating brains at 4C in rabbit anti-GFP (1:250) and by FITC-coupled goat-anti rabbit secondary antibody (1:500). Neuropil staining was achieved by incubating brains in a1:50 dilution of NC82 mouse antibody and with a TRITC-coupled goat anti-mouse antibody. Brains were imaged with a Leica confocal microscope.

Cell Culture and Co-Immunoprecipitations

Drosophila S2-Gal4 cells were maintained at 26°C, in Schneider medium (Gibco BRL) with 10% Fetal Bovine Serum. Constructs were made using Gateway

cloning (T. Murphy, Carnegie, Baltimore, MD) and clonase (Invitrogen), and transfected using standard calcium chloride protocol. Stable transfections were generated with pCoHygro (Invitrogen) and maintained in the presence of 22mg/ml hygromycin in the medium. Anti-*Arf6* dsRNAi was generated using the Megascript T7 kit (Ambion), and cells were treated daily with 3µg dsRNAi for three days. Serum starved cells were treated with insulin (Sigma-Aldrich) or wortmannin (LC Laboratories) for 30 min prior to harvesting. For pull-downs, cells were washed in PBS, and lysed in IP buffer (50mM Tris-base pH=7.4, 50mM sodium chloride, 1% TritonX-100, 4mM magnesium chloride, protease inhibitor mixture, Roche Molecular), incubated for four hours with FLAG-beads (Sigma- Aldrich), washed in PBS with equal volume of 2x Lämmli sample buffer added before western analysis.

Antibody Techniques

Immunohistochemistry was performed on whole mount brains as described (Wu and Luo, 2006), using anti-GFP 1:250 (Invitrogen), anti-bruchpilot (nc82, 1:50), and FITC- and TRITC-labeled secondary antibodies (1:500, Sigma-Aldrich). The nc82 antibody developed by Erich Buchner, was 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 52242. Western blots were performed using anti-*Arf6*

antibody (1:1000, Sigma-Aldrich), anti-*Arfip* (1:1000, (Chang et al.)), anti-*S6K* (1:1000 a gift from Thomas Neufeld, University of Minnesota), anti-P-*S6K* (1:2000, Cell Signaling Technology), anti-GFP (1:5000, Invitrogen), anti- FLAG (1:1000 Sigma-Aldrich), anti-actin (1:400, Sigma-Aldrich), and anti-HRP secondaries (1:5000, Cell Signaling Technology), and visualized using enhanced chemiluminescence (Amersham). Densitometry was conducted using Adobe Photoshop Creative Suite 3 (CS3) analysis tool. We report ratio of mean grey value relative to background for an average of three separate blots probed with both anti-*S6K* and anti-P-*S6K*.

Statistics

Statistical significance was computed using Student's t tests assuming equal variance or one-way analysis of variance (ANOVA) tests, followed by post-hoc Tukey testing using GraphPad Prism software. The standard error of the mean (SEM) is represented by error bars in all experiments.

Discussion

Arfip Functionally Connects the Rho-Family to Arf6 GTPase to Regulate Ethanol Sedation Behavior

A study by Rothenfluh et al. (2006) determined that white rabbit (whir), a mutation in the X-linked gene RhoGAP18B, induces behavioral resistance to alcohol sedation. In most organisms, low alcohol doses induce hyperactivity while high levels induce sedation. Mutants of RhoGAP18B exhibit both, a reduction in hyperactivity and decreased sedation in response to acute alcohol exposure. To identify RhoGAP18B interacting genes, a modifier screen was performed which resulted in the isolation of mutations in Arfip (a.k.a. POR1 or Partner of Rac1) and Arf6.

Arfip and Arf6 mutants exhibit behavioral ethanol sensitivity on their own (Fig c and d) and can genetically interact with RhoGAP18B by suppressing its ethanol resistance phenotype (Fig 1a and b). Compared to Arf6 and Chpd double mutants, double loss of function mutations in Arf6 and Arfip are non-additive (Figure 1e and f), indicating that Arf6 and Arfip function in the same pathway, and suggests that this pathway may be independent of cAMP.

Epistasis experiments were then performed to establish the hierarchical relationship between components of the Rho and the Arf signaling pathways (Fig 4 c, d, and e). RhoGAP18B inhibits rac1 in vitro and acts via Rac1 in vivo (Rothenfluh et al., 2006). Over-expression of Rac1 constitutive GTP-locked

mutants produces resistance to ethanol sedation, while Rac1 GDP-locked mutant over-expression produces sensitivity to ethanol sedation. Mutations in RhoGAP18B or expression of constitutive Rac1-GTP in Arf6 mutants results in strong ethanol sedation (Fig 4d), while CNS expression of Arf6 overcomes the ethanol sensitivity of Arfip mutants by conferring strong resistance to the intoxicating effects of ethanol (Fig 4e). Results indicate that Arf6 mediates naïve ethanol resistance downstream of Rac1 and Arfip.

Some studies indicate that Arfip binds to Rac1.GTP (Van Aelst et al., 1996), but other studies have shown it bind Rac1.GDP (Shin et al., 2001), and yet another study showed that Arfip binds to both Rac1.GTP and GDP (Tarricone et al., 2001). Arf6 and Rac1 do not physically interact, however, Arfip has been shown to bind to Arf6.GTP but not GDP (Shin et al., 2001; Kanoh et al., 1997; D'Souza-Schorey et al., 1997). To investigate how the Rho pathway connects to Arf signaling, pull-down experiments in Drosophila cells were performed (Figure 5). Results of these experiments demonstrate that Arfip interacts preferentially with GTP forms of Rac1 and Arf6 GTPases, thus acting as a molecular link that mediates Rac1 (Cherfils, 2001) signaling to Arf6 (Boshans et al., 2000; Tarricone et al., 2001; Koo et al., 2007; Palamidessi et al., 2008) in the regulation of behavioral sensitivity to ethanol sedation.

Rac1 and Arf6 co-localize on recycling endosomes and on the plasma membrane, and translocation of Arf6 and Rac1 to the cell surface is produced by

stimulation of Arf6 in cell culture experiments (Radhakrishna et al., 1999). Rac1, Arfip, and Arf6 are known regulators of the actin cytoskeleton (Zhang et al., 1999; Schafer et al., 2000; Santy and Casanova, 2001) particularly regulators of peripheral actin and membrane dynamics (Palamidessi et al., 2008), and it may be possible that Arfip may also be the mediator of Rac1 and Arf6- dependant changes in cellular architecture (Santy and Casanova, 2002). Changes in actin cytoskeleton are known to affect membrane trafficking (Galleta and Cooper, 2009; Radhakrishna et al., 1999; Schafer et al., 2003). Thus, impairment in actin dynamics disrupts endocytosis (Galletta and Cooper, 2009) and receptor recycling (D'Souza Schorey et al., 1995; Radhakrishna et al., 1999), and it also impairs Arf6 cellular re-distribution at the cell surface and recycling compartments (D'Souza-Schorey et al., 1998).

Because Arfip does not encode a CRIB Rac-effector, or a GEF domain, we suggest that an Arf-GEF turns on Arf6 in recycling endosomes (Maranda et al., 2001), as well as at the plasma membrane when a signal from the activated receptor activates scaffolding proteins critical for Arf6 recruitment at the cell surface. Once activated, Arf6 binds Arfip. Arfip functions in vesicle formation (Peter et al., 2004), and then recruits Rac1, which functions in actin dynamics to regulate membrane trafficking at the plasma membrane. It remains unclear whether the mechanism by which these GTPases control acute ethanol-induced behaviors is endocytosis and recycling directly, or through the actin cytoskeleton.

Arf6 and Arfip are known to function in vesicle formation (Peter et al., 2004; Honda et al., 1999), and independently, Arfip can regulate membrane curvature through its Bin/Amphiphysin/Rvs (BAR) domain, which binds to Rac1 (Peter et al., 2004; Habermann, 2004). Mechanisms underlying cellular architecture and cytoskeletal dynamics are essential for intracellular transport and endocytosis, and since Arf6 can regulate mu-opioid receptor endocytosis and recycling (Rankovic, 2009), it may also function similarly in InR trafficking.

Arf6 Regulates InR signaling to Control Behavioral Ethanol Responses

Some studies have shown that Arf6 functions in mammalian InR signaling (Millar et al., 1999; Li et al., 2007), though several other reports did not find a role of Arf6 in InR signaling (Yang and Mueckler, 1999; Lawrence and Birnbaum, 2001; Bose et al., 2001). Two recent papers suggest that Cytohesin2 (Arf6-GEF) is recruited upon InR activation, through scaffolding protein CNK1, which activates PIP5 kinase and IRS-1 (Hafner et al., 2006; Lim et al., 2010). Arf6 RNAi disrupts insulin-induced AKT-P, while expression of constitutive-active Arf6 (Q67L) increases AKT-P even in the absence of insulin (Lim et al., 2010).

Our data demonstrate that Arf6 is a critical regulator of InR signaling in Drosophila cells and in vivo. Genetic epistasis experiments indicate that constitutively active InR fails to signal in Arf6 mutants (Fig 5a), though activation of its effector S6K rescues the ethanol hypersensitivity of Arf6 mutants (Fig 5b).

These experiments show that Arf6 functions downstream of InR but upstream of S6 kinase. Cell culture experiments were performed to determine whether Arf6 induced signaling would be sensitive to changes in PI3K. Results showed that Arf6 regulates S6K-P downstream of InR, but in a PI3K dependent manner. Constitutive active Arf6 increases S6K-P and can also enhance insulin-induced S6K-P. Pharmacological inhibitor of PI3K, wortmannin, diminished both insulin and Arf6-induced S6K-P (Fig 5f). Additionally, Arf6 knock-down reduced baseline and insulin-induced S6K-P, and the sharp decreases in S6K-P produced by reduction of Arf6 did not enhance that of wortmannin (Fig 5E). Indicating that Arf6 regulates InR signaling upstream of PI3K.

Complete loss of InR produces lethality (Fernandez et al.,1995), as is with S6K loss, but occasionally, there are survivor mutants with small-body size (Montagne et al 1999). Partial loss of function mutations in other InR signaling components also produce small body size (Montagne, 1999), and ablation of IPC's in flies, which decrease InR signaling, produce diabetes mellitus-like pathophysiology (Rulifson et al., 2002). Arf6 mutants are viable and morphologically normal (data not shown), with male infertility being the only overt phenotype (Dyer et al., 2007). Some reports in adipocytes indicate that Arf6 regulates insulin's effects on Glut4 plasma membrane translocation (Millar et al., 1999), though another report that Arf6 regulates trafficking of Glut4 in response to endothelin but not insulin (Bose et al., 2001). These data supports the

hypothesis that the role of Arf6 in InR signaling is restricted to some functions of insulin function, but not all.

In our cell culture experiments, we show that serum-starved cells have baseline S6K-P, which is sensitive to changes in Arf6, while Arf6-induced increase in baseline S6K-P is abolished by wortmannin (Figure 5 d, e, and f). This suggests that Arf6 may function to maintain temporary baseline InR signaling in times of Insulin scarcity. It has been shown in adipocytes that plasma membrane recruitment of Cytohesin2 is dependent on PI3K produced PIP_3 (Venkateswarlu et al.,1998), which suggests that InR signaling to PI3K occurs first and subsequently Arf6 is recruited via Cytohesin2 for signal amplification. In the absence of this Arf6 feed-forward mechanism, sufficient InR signaling might thus occur maintain viability in the absence of Arf6, and explain why loss of InR and S6K result in lethality, but loss of Arf6 does not.

While too much insulin can repress InR signaling (White, 2003), the and it is only released when blood glucose levels surge or in relation to signals stemming from body adiposity (Plum et al., 2005; Bagdade et al., 1967; Polonsky et al., 1988). Because of this, surface InR's must be sensitized to insulin. Either the inability of InR's to sensitize to insulin (i.e. insulin resistance), or failure to maintain baseline InR signaling, which may in turn help to sensitize InRs via a feed-back mechanism (Puig et al., 2004), constitute pathological conditions that are at the heart of diseases, such as diabetes type II (Bar, 1983), and since Arf6

functions to amplify InR signal, it might offer a protective role against insulin related-morbidity.

Neuronal S6 Kinase Mediates Acute Ethanol Behaviors Downstream of Arf6

InR signaling changes regulate ethanol-induced behaviors (Corl et al., 2005; Eddison et al., 2011), and ethanol exposure in turn alters InR signaling (McClure et al., 2010; He et al., 2007). The acute and chronic effects of ethanol on InR signaling have been documented by several studies. In consensus, low ethanol doses increase insulin signaling, which increase insulin sensitivity in humans (Facchini et al., 1994), and high ethanol levels decrease InR signaling (He et al., 2007), using as measures AKT phosphorylation, or ethanol-induced acute insulin-resistance in humans (Shelmet et al., 1988). Our data shows that S6K, an InR and Arf6 effector, regulates naïve ethanol sedation.

Acute ethanol sedation alters neuronal S6K-P in a dose-dependent manner. Low ethanol levels increase S6K-P, and high doses, which confer total sedation, completely abolish S6K-P (Fig 6). Thus, ethanol-induced S6K-P changes and ethanol-induced sedation strongly correlate *in vivo*. Arf6 mutants are completely devoid of neuronal S6K-P and ethanol levels that increase S6K-P in wild-type flies, fail to increase S6K-P (Fig 6). This data suggests that the S6K-P loss contributes to ethanol's intoxicating effects, and the lack of S6K-P in the ethanol-sensitive Arf6 mutants, pre-disposes them for early intoxication.

How ethanol affects S6K-P is unknown, as it is also not known whether ethanol itself can directly interact with the InR receptor at low millimolar concentrations and affect signaling (Seiler et al., 2000). Ethanol inhibits receptor tyrosine autophosphorylation at pharmacologically relevant doses (Banerjee et al., 1998); Luo and Miller, 1999; Resnicoff et al.,1993). It is unclear whether ethanol disrupts InR tyrosine kinase (RTK) auto phosphorylation by interacting directly with the receptor or factors in the receptor complex. Seiler et al., (2000) found that 150 mM ethanol non-competitively inhibits the InR tyrosine kinase domain. The authors narrowed ethanol's direct effects to the carboxy terminal end of InR. While ethanol disrupted ligand-dependent InR tyrosine-P, ethanol-reduction of kinase activity was half maximal at roughly 25 mM ethanol.

Ethanol can also impair insulin signaling in tissues other than the brain, such as skeletal (Kumar et al., 2002) and cardiac (Lang et al., 2000) muscle. Though the present dissertation focuses on the role of InR signaling and the effects of ethanol in the nervous system, it is recognized that ethanol might affect other tissues in a distinct manner. Little is known about the way ethanol affects InR/S6K signaling in the nervous system, however, in skeletal muscle, ethanol does not appear change AKT, IRS-1, or InR tyrosine phosphorylation levels, but ethanol does impair the ability of either insulin or insulin growth factor 1 (IGF-1) to phosphorylate S6K sites T389 and T421/S424 (Kumar et al., 2002). Lang et al. (2004) showed that intra-peritoneal ethanol injection prevented the ability of IGF-

1 to phosphorylate S6K and this effect lasted up to 8 hours, even in rats that received 4-methylpyrazole. Thus suggesting that ethanol's inhibitory effects on IGF-1's ability to phosphorylate S6K occurs independently of ethanol metabolism. Interestingly, ethanol did not reliably prevent IGF-1 from phosphorylating 4EB-P1, another InR effector which functions in a parallel pathway to S6K. This data suggests that ethanol might affect insulin-induced translation of selected mRNAs and protein synthesis (Proud, 2006).

At the same time, since ethanol affects InR autophosphorylation (Seiler et al., 2000), the InR might not signal downstream resulting in S6K not being activated via PI3K/AKT, although it is also recognized that certain steps in the InR pathway are not obviously affected by ethanol such as the adapter protein IRS-1 and PDK1 (Kumar et al., 2002). Studies in skeletal muscle only tested for acute ethanol intoxication, while here I present data for ethanol's dose dependent effects on behavioral ethanol sensitivity and S6K-P, both of which are strongly correlated to one another (See Fig 6).

Chronic ethanol uptake has been shown to stimulate secretion of various hormones such as insulin and thyrotropin releasing hormone (TRH) in rat pancreatic islets (Benický et al., 2000). Several days of ethanol drinking can also increase insulin/InR binding in rat adipose and liver tissues, but not in skeletal muscle (Macho et al., 2003). Just as it is unclear how ethanol suppresses S6K-P, It is also unclear how lower acute ethanol doses increase S6K-P (Fig. 6), no data

has shown that acute ethanol stimulates insulin peptide release, which would have provided some account for our observation.

Explaining why Arf6 mutants are devoid of neuronal S6K-P is more difficult, particularly since loss of S6K results in lethality, while Arf6 mutnats are viable. In order for S6K to be fully active it must be phosphorylated at seven serine/threonine residues (Kumar et al., 2002), and the absence of Arf6 may prevent phosphorylation of all or some of these sites, causing diminishing S6K-P. Alternatively, IRS-1 has been shown to be a substrate of S6K via a feed-back mechanism (Harrington et al., 2004). Therefore, it may be possible that lack of Arf6 might reduce S6K-induced IRS-1 activation, resulting in downstream signaling rundown. Diminished signaling by IRS-1 cannot be amplified in the absence of Arf6, thus exacerbating this cycle. However, this is unlikely since lack of S6K produces lethality, although it may be worth investigating whether losing S6K in specific tissues such as the brain, may not produce lethality.

The fact that ethanol alters acute S6K-P levels and the lack of S6K-P strongly correlates with ethanol intoxication, ethanol-induced effects on S6K-P might be a better readout for why Arf6 mutants are so sensitive to ethanol's intoxicating effects. Therefore, I propose that ethanol affects in vivo S6K-P by partially altering insulin induced InR signaling, which is dependent on Arf6 (Fig 6), although it is unclear whether acutely, ethanol affects Arf6 GTP status.

InR/Arf6/S6K Signaling May Function in Synapse Size and Number to Control Ethanol-Induced Behaviors

InR signaling is essential to many nervous system functions, including neuronal growth, synaptogenesis, and cognition. The InR receptor is ubiquitously expressed in the mammalian brain (Havrankova et al., 1978), and in flies, the InR expression profile becomes more restricted in adult *Drosophila* NS (Fernandez et al., 1995; Gorczyca et al., 1993). Ellipsoid body InR overexpression produces neuronal enlargement and increased synaptogenesis (Martín-Peña et al., 2006). At the neuromuscular junction (NMJ), over expression of InR effectors PI3K, AKT or S6K enhance bouton number and mini frequency (Martín-Peña et al., 2006; Knox et al., 2007; Howlett et al., 2008).

A recent report in Xenopus tadpoles revealed that InR expressing optic tectal neurons have decreased synaptic density, AMPA mEPSC's, and abnormal dendritic arborizations, but normal release probability (Chiu et al., 2008). Unpublished results from Chang et al. show that Arfip mutants have reduced synaptic growth at the NMJ, while increase in growth is produced by Arfip over-expression at the NMJ. Our Arf6 mutant brain immunostaining analysis revealed no gross structural defects (Fig. 2g), still Arf6 mutant brains might have subtle defects only detectable at the ultrastructural level (i.e synapse size and/or number. A recent study revealed that synapse number is a factor affecting behavioral sensitivity to the intoxicating effects of ethanol (Eddison et al., 2011).

Recent evidence shows that Arf6 regulates long-term synaptic depression (LTD) (Scholz et al., 2010), and many of its GEF regulators have been shown to interact with the postsynaptic density (PSD) or post-synaptic specializations (Sakagami, 2008) of both excitatory (Sakagami et al., 2008) and inhibitory synapses (Fukaya et al., 2011) suggesting that Arf6 functions in synaptic plasticity. Homeostatic plasticity affects synapse size, with larger synapses having greater release probability than smaller ones, particularly since synapse size reflects the size of the readily-releasable vesicle pool (Murthy et al., 2001).

Consistent with data showing that Arf6 functions in synaptic plasticity, Arf6 is enriched in synaptic plasma membrane fractions and when activated, Arf6 induces AP-2/clathrin coat formation in synapses via PIPKIγ in rat brain-derived cytosol and LP2 membranes (Krauss et al., 2003). In addition, Arfip mutants have impaired quantal content at the NMJ (Chang et al. unpublished results), thus in addition to a possible defect in synapse number, InR/Arf6/S6K signaling may also be involved in homeostatic synaptic plasticity and synapse size. Given that Arf6 may have crucial roles in excitatory and inhibitory synapses, it is feasible to envision that chronic alcohol abuse may alter Arf6 function at synapses and may contribute to the alcohol withdrawal seizures in mammalian models and in alcoholics.

Arf6 Regulation of Membrane Trafficking

Ras family-related members of the Arf family of GTPases, primarily Arf1 and Arf6, have been implicated in receptor endocytosis and recycling (D'Souza-Schorey et al., 1995; Peters et al., 1995; Kumari, et al., 2008). Arf6 and Arf1 have overlapping multifarious functions, perhaps due to their amino acid sequence similarity. Despite being expressed in all tissues/cell types tested they have distinct in vivo localizations and functions (Peters et al., 1995 Al-Awar et al., 2000; Cavenagh et al., 1996; Song et al., 1998). Arf1 localizes at the Golgi system and functions in cytosolic coat protein assembly, including adapter proteins, in order to regulate membrane trafficking within the ER-Golgi complex. Conversely, Arf6 has been shown to regulate receptor trafficking from the plasma membrane to a recycling endosome, via a tubular compartment typically associated with Arf6 (Brown et al., 2001; Radhakrishna et al., 1997) and this transport of receptors across endocytic steps is dependent on Arf6 nucleotide exchange (Peters et al., 1995).

Arf6 is a regulator of the actin cytoskeleton, and actin dynamics directly affect endocytosis and recycling (Boulant et al., 2011). For this reason, demonstrating that Arf6 has a direct and separable role in intracellular trafficking has been difficult. Some reports show that activated Arf6, forms actin-related protrusions at the plasma membrane (Radhakrishna et al., 1996) and also mediates Rac-induced membrane ruffling (Radhakrishna et al., 1999). By

contrast, expression of wild-type Arf6 in conjunction with treatment of actin Polymerization inhibitor cytochalasin D, or expression of dominant-negative Arf6 (Arf6T27N) alone, inhibits recycling to the plasma membrane (Radhakrishna et al., 1997) and Rac-induced membrane ruffling (Radhakrishna et al., 1999).

A paper by Al-Awar and colleagues (2000) showed that an Arf chimera, containing the amino half of Arf1 and the carboxy half of Arf6, which inhibited Arf6-induced protrusions, did not affect either Arf6-mediated membrane trafficking or Rac-mediated ruffling. Together, this data suggests that Arf6-mediated membrane trafficking is independent of actin-containing protrusions, and it may involve Rac-induced ruffling. The extent to which Arf6 requires Rac induced ruffling for endocytosis is unclear, but a recent paper has shown that via calmodulin, Rac1 can regulate Arf6-dependent endocytosis in HeLa cells (Vidal-Quadras et al., 2011).

How Arf6 controls membrane trafficking remains unclear. A number of studies show that Arf6.GTP promotes internalization and recycling, while Arf6.GDP inhibits it (Rankovic et al., 2009), other studies have either found the opposite (Sannerud et al., 2011; Naslavsky et al., 2003) or that Arf6.GTP has no effect on endosomal mechanisms, while inactive Arf6 or ARNO (Arf6-GEF) promote endosomal redistribution at the plasma membrane in hippocampal neurons (Hernandez-Deviez et al., 2007). Yet other reports indicate that expression of Arf6 GTP-locked mutant (Arf6 Q67L) prevents Transferrin (Tf)

receptor recycling by sequestering internalized cargo in vacuole-like compartments, which lack Rab5, EEA1, Rab4, or Rab11 (Chesnau et al., 2012), while Arf6T27N promotes endocytosis, but inhibits recycling (Brown et al., 2001; Sannerud et al., 2011). One could argue that differences in experimental findings may reflect the distinctive function Arf6 plays in various cell-types, but the discrepant results obtained by various research groups using the same cell type, may be more of a result of differences in experimental protocols and reagents used.

Arf6 involvement in clathrin-mediated endocytosis (CIE) and recycling is also controversial. In Chinese Hamster (CHO) cells Arf6 partially localizes with Tf receptor-associated endosomes (D'Souza-Schorey et al., 1998). In HeLa cells Arf6-dependent endocytosis appears to be mediated independent of clathrin internalization (Radhakrishna et al., 1997 Naslavsky et al., 2003; Sannerud et al., 2011). However, Arf6 exocyst complex effector sec10, which inhibits Arf6 Q67L-induced cell spreading in NRK cells, reduces Tf recycling when knocked-down via RNAi in HeLa cells, suggesting that Arf6 may function in clathrin-dependent pathways (CDP) (Prigent et al., 2003; Krauss et al., 2003; Montagnac et al., 2011). Moreover, another paper in HeLa cells showed that Arf6-Q67L inhibits Tf endocytosis but not recycling, and this mechanism is mediated via Arf6-induced recruitment of AP-2 adapter to the cell surface (Paleotti et al., 2005).

It may be indeed possible that Arf6 under specific conditions or cell types regulates both CIE and CDE via distinct GEFs, particularly since distinct GEFs have been shown to either promote or inhibit internalization (Franco et al., 1999; Tanabe et al., 2005). Arf6 has been shown to be expressed in clathrin coated pits and structures. Although GTP inactive Arf6 mutants (Arf6T44N and Arf6T27N) failed to associate with clathrin-dependent structures or affect CDE, both the fast cycling (Arf6T157N) or the GTP-locked Arf6 Q67L mutants associated with clathrin coated structures, suggesting that GTP hydrolysis is required for Arf6 interaction with clathrin coated pits and structures (Montagnac et al., 2011)

Despite data showing Arf6 involvement in CDE, Arf6 is usually associated with CIE (Donaldson et al., 2009). CIE has been difficult to study given the lack of protein markers. More recently, proteins have been identified that do not interact with AP2/clathrin associated endocytosis, such as major-histocompatibility class I (MHCI), interleukin 2 receptor α subunit (Tac), and the GPI-anchored CD59 (Naslavsky et al., 2003; Eyster et al., 2009) which can be internalized via Arf6 and have been used to study CIE (Naslavsky et al., 2003; Grant and Donaldson et al., 2009; Donaldson et al., 2009; Sannerud et al., 2011). The truth of the matter is that the stability of CDE may depend on the integrity of CIE and vice versa. Both pathways are parallel routes of entry into the cell, and may share downstream components or ligand-dependent factors at the plasma membrane, which are critical for internalization processes. Evidence for this comes from a

study in which Arf6 knockdown via siRNA, affected ligand dependent endocytosis independent of the route of entry (Houndolo et al., 2005; Donaldson et al., 2009).

Early steps of CDE are more regulated than early steps in the CIE, but recycling-related steps are many times more regulated than in CDE. Therefore, disruption of critically regulated steps in either mode of entry may consequently affect the other. In support of this notion, Sannerud et al. (2011) discovered that modulation of Arf6-mediated internalization of β-site cleaving enzyme 1 (BACE1), which occurs via CIE, affects the clathrin-dependent processing of amyloid precursor protein and amyloid β formation. Therefore, subcellular modulation of Arf6 via CIE may be a novel route to modulate entry via the CDE.

Many reports indicate that Arf6-Q67L forms vacuoles or macropinosomes, which are molecularly characterized by increase in Arf6 stimulated PIP_2 via PLD – induced activation of PIP5K. Phosphoinositide alteration induces changes in lipid composition, and under constitutive Arf6 activity, elevated PIP_2 levels lead to abnormal production of vesicle membranes given the fusion of PIP_2 positive membranes, and membrane ruffling at the plasma membrane (Brown et al., 2001). Under normal Arf6.GTP conditions, levels of PIP_2 persist until inactivation of Arf6 occurs. Arf6Q67L-induced vacuoles lack PIP_3 and EEA1. Loss of PIP_2 is potentially caused by a PIP5-phosphatase or the activity of other molecules like Phospholipase C (PLC) (Donaldson et al., 2009), and it enables endosomal transport and fusion into PIP_3 and EEA1 positive endosomes. While this vesicle

fusion depends on Arf6 GTP hydrolysis (Sannerud et al., 2011; Donaldson et al., 2009; Brown et al., 2001).

An important question that remains unanswered is whether Arf6-mediated recycling requires Rab-positive endosomes, or whether an Arf6 recycling endosome is sufficient for reinsertion of recycled material back to the surface. It has been proposed (Naslavsky et al., 2003) that early Arf6 endosomes fuse with an Arf6-associated tubular structure that contains PIP_3 and EEA1. This step is both dependent on Arf6 GTPase hydrolysis and PI3K.

From this tubular sorting structure material to be recycled is transported either to an Arf6 recycling endosome or into a Rab5 and EEA1 positive endosome, which functions via CDE. In the latter, Tac chimeras have been generated to internalize via CDE. These chimeric proteins converge with material internalized through Arf6 in these Rab5 and EEA1, suggesting that Rab5 functions as a relay station at the CIE/CDE interface (Naslavsky et al., 2003). Reasons for this are unknown, although it has been shown that Arf6-internalized material is transported from Rab5 endosomes into late endosomes and subsequent degradation, while Tac chimeras can be successfully recycled via clathrin dependent recycling compartments.

Various reports indicate that the InR internalizes via CIE which does not require dynamin (McClain and Olefsky, 1988; Smith and Jarrett, 1990), although is has been shown that InR endocyosis in dependent on dynamin, implying a

requirement for CDE (Ceresa et al., 1998). Consistent with the InR and CDE connection, Arf6 has also been found to facilitate dynamin-mediated endocytosis (Palacios et al., 2002). Whether or not Arf6 is involved in InR internalization is an open question currently being investigated in our laboratory, preliminary results indicate that Arf6-Q67L can internalize the InR at a faster rate than wild type Arf6, while Arf6 knockdown-impairs insulin-induced InR internalization. Future experiments will establish whether Arf6 endocytosis and recycling of InR turns out to be the key mechanism by which InR/Arf6/S6K controls the molecular processes underlying acute and consumatory ethanol behaviors.

Arf6 Controls InR Signaling and Acute Ethanol Behaviors via Endocytosis and Recycling of InR: A Proposed Model

Signal transduction mechanisms convey information from the extracellular environment to the interior of the cell. Internalization of extracellular stimuli induces the orchestration of intrinsic molecular events that culminate in physiological and behavioral responses. The ability of a cell to recognize stimuli from the extracellular milieu is directly related to receptor density at the plasma membrane. Upon receptor activation by its ligand, post-translational modifications take place, which recruit adapter molecules to aid in downstream signaling and in the internalization process itself (Platta and Stenmark, 2011).

Early endosomes are the first relay station for internalized receptors, thus surface receptor activation is not the exclusive site of signal transduction (Sorkin and Zastrow, 2009). Ligand binding to the endocytosed receptor persists in the early and intermediate stages of endosomal sorting, allowing the receptor to signal on route to other endosomal compartments (Khan et al., 1989; Burgess et al., 1992; Sigismund et al., 2005; Calebiro et al., 2009). Because distinct compartments contain distinct intramolecular mechanisms for signaling, the receptor can activate proteins in a canonical manner, but also other molecules that are not normally activated during surface signaling (Platta and Stenmark, 2011).

Endosomal compartments vary in their lipid and protein make up, as well as in their pH (Grecco et al., 2011). Acidification leads to ligand-receptor decoupling and this turns off signaling. Receptors can be either retained in a specific compartment, or shuttled into a recycling or a late endosomal compartment via highly regulated vesicle budding and fusion mechanisms. Although it is generally thought that late endosomes are the final station prior to lysosomal degradation, reports show that they can contribute to signaling (Lu et al., 2009; Poteryaev et al., 2010).

GTPases of the Rab family are critical regulators of endosomal sorting, and like Rab proteins, Arf6 GTPase functions in membrane trafficking and can effectuate separable and distinct mechanisms in signaling and endocytosis in its

own right. Endocytic mechanisms are essential to signaling, but how signaling contributes to endocytosis remains unknown. Hence, I postulate that Arf6 regulates InR signaling via endocytosis and recycling of its InR receptor, but in addition, it plays a functional role in InR signaling independent of InR endocytosis (See Fig 8).

InRs are RTK membrane proteins. InRs are composed of two extracellular α subunits and an intracellular β subunit, which is linked by two disulfide bonds (Carpentier et al, 1992; Maegawa et al., 1988). Canonically, insulin activates InR at the plasma membrane upon binding, causing it to undergo autophosphorylation (Hari et al., 1987), endosomal internalization, and initiation of a signaling cascade. Preventing InR endocytosis does not inhibit its autophosphorylation nor did it inhibit AKT phosphorylation or that of its substrate, IRS-1. However, it did prevent the activation and phosphorylation of the adapter molecule src homology 2 containing (SHC) adapter protein. (Giudice et al., 2010; Biener et al., 1996; Ceresa et al., 1998; Hamer et al., 2002), suggesting that activation of these signaling components occurs at the plasma membrane.

InR signaling can occur and InRs can still be internalized (in smaller numbers) in insulin free conditions, signifying that the biological purpose of InR internalization, or any other receptor is not clear-cut. And although it may be possible, it is highly unlikely that the few InRs that get internalized in the absence

of insulin contribute to baseline InR signaling. Many suggestions have sought to explain the function of endocytosis in InR signaling, including: the removal of insulin from the circulation by digestion, to promote or enhance InR signaling itself, and as a mechanism to turn “off” InR signaling in response to insulin binding via ligand-receptor decoupling (Brännmark et al., 2010).

Data described in the present dissertation shows that behavioral ethanol responses require Arf6 and InR signaling. I will now propose a model to explain how mechanistically, Arf6 controls ethanol-induced behaviors via the regulation of InR signaling. Endocytic regulation of InR in *Drosophila* cells has not been investigated, and the proposed model will accommodate evidence of how Arf6 mediates endocytosis and recycling, and how InR is internalized based on evidence available from other cell-types. The view that InR internalization is necessary, but not sufficient to increase InR signaling is favored in this working model, as well as the hypothesis that InR recycling functions to promote InR signaling by refurbishing the supply of InR receptors to the plasma membrane for continued rounds of signaling.

Blocking InR internalization and recycling via Arf6 RNAi or over expression of Arf6.GDP-locked mutant (Sannerud et al., 2011) will be detrimental to InR signaling. Indeed, a report by Rizzo et al (1999) showed that inhibitor of Arf-GTPases Brefeldin A (BFA) inhibits InR internalization via CDE mechanisms. From the perspective that Arf6 functions in InR recycling, it can be argued that

receptor density stemming from the remaining surface InRs (or the un-internalized InRs), paired with InR provision from the Golgi (Posner et al., 1978), may be insufficient to keep up with the impinging demand for InR signaling, as it may be required during an acute ethanol exposure. Thus, it should be investigated whether an Arf6 GEF is present at the recycling endosome or at the plasma membrane to enable Arf6-mediated reinsertion of InRs into the cell's surface.

Based on previous evidence demonstrating that inhibition of InR internalization does not affect downstream signaling (Hamer et al., 2002), then the lack of insulin-induced InR signaling produced by Arf6 RNAi in S2 cells, or depletion of S6K-P in Arf6 mutants could not be fully accounted for, if Arf6 affected InR signaling exclusively through endocytosis of its cognate receptor. It is predicted that since constitutive active Arf6 forms vacuoles, the internalized coupled insulin-InR will be retained in Arf6 vacuole structures where it can continue to signal and activate other molecules, though most of the observed InR signaling is hypothesized to stem from plasma membrane InR activation, and most importantly through Arf6 mediated feed-forward signal amplification, thus for this model, I argue, that Arf6 has a dual and separable direct role in InR signaling, via InR endocytosis and the InR signal cascade via PLD and PIP5K (Brown et al., 1993; Moritz et al., 1992).

If the above hypothesis is true, InR activation eventually leads to Arf6 GTP to operate at the plasma membrane perhaps via a GEF. Whether or not insulin stimulation leads to Arf6 GTP nucleotide exchange, or Arf6 is recruited through alternative mechanisms requires verification. Consistent with this view, since over expression of Arf6 Q67L increases InR signaling, it is hypothesized that it will increase InR internalization. GTP hydrolysis appears to be imperative for vesicle sorting from an early Arf6 compartment to either a tubular structure associated with CIE, or a Rab5/EEA1 positive endosome (Sannerud et al., 2011, Naslavsky et al., 2003), suggesting the presence of an Arf6-GAP in this early compartment, and that internalization from the plasma membrane into an early Arf6 compartment requires Arf6 GTP.

Arf6 alters lipid composition and forms protrusions and ruffling at the plasma membrane. Thus, an over abundance of Arf6 changes the membrane's fluidity causing an increase in the kinetics rate of InR internalization (Evans and Bowman, 1992). In addition, Arf6-induced protrusions have been shown to induce internalization (Donaldson et al., 2009), and too much Arf6 contributes to InR endocytosis and signaling through this process.

Arf6.GDP or the absence of Arf6 does not affect BACE1 internalization, but it affects BACE1 recycling (Sannerud et al., 2011). If activated Arf6 is required for endocytosis from the plasma membrane into an Arf6 endosome, then

I hypothesize that in *Drosophila* cells, little or no InR endocytosis will be induced by Arf6.GDP or Arf6 knock-down. This hypothesis is consistent with our data showing that lack of Arf6 impairs InR/S6K signaling.

InRs are internalized through clathrin-coated pits, and that Arf6 may function in this route of internalization in some cell-types. The preponderance of the evidence favors the notion that Arf6 mediates CIE. In the present dissertation I show that Arf6 regulates InR signaling (Fig 5 and 6). It has been long documented that endocytosis and signal transduction are connected, and together these data would suggest that Arf6 regulates InR signaling through CIE of InRs. In such instance, inhibiting CDE pharmacologically (i.e. dynasore), or through expression of dynamin mutants should only affect Tf internalization, but not InR endocytosis or InR signaling for that matter. Similarly, inhibition or depletion of Arf6 should only affect InR endocytosis, but not Tf internalization.

Ethanol's chronic (Rifkin et al., 1983; Tuma et al., 1991) and acute effects on InR endocytosis (Fawcett et al., 1993) have been investigated. In general, ethanol impairs the rate of InR internalization, but it does not affect insulin-receptor binding (Fawcett et al., 1993). It remains unclear whether ethanol may affect InR endocytosis and signaling directly, or through Arf6.

How acute ethanol exposure affects Arf6, has not been investigated, although chronically ethanol increases neuronal Arf6 expression. In a study using hippocampal-derived neurons from developing rats, Marin and colleagues (2010)

found that while chronic exposure to ethanol down regulated endocytosis-related proteins including Rab5, Rab11, and EEA1, it caused Arf6 upregulation. Similarly, microarray analysis of nucleus accumbens extracts of ethanol-preferring rats showed a slight but significant increase in Arf6 RNA expression levels (Bell et al., 2009; Saito et al., 2002). Finally, ethanol causes major changes in cell morphology through alterations in actin cytoskeleton dynamics (Allansson et al., 2001; Offenhauser et al., 2006). So it may be that acutely, toxic ethanol levels might cause defects in actin depolymerization at the plasma membrane resulting in impairment of InR endocytosis and InR/S6K signaling.

A proposed mechanism by which ethanol inhibit S6K-P is via Arf6-effector PLD, which is acutely suppressed by ethanol in various tissues (Alling et al., 1984; Hoek et al., 1992; Rydzewska et al., 1996). Insulin can activate PLD, which generates PA by hydrolyzing phospholipid substrate, phosphatidylcholine (Rizzo et al., 1998). However, Insulin induced activation of PLD is inhibited by treatment with Arf-inhibitor BFA (Rizzo et al., 1998). PA is a second messenger that can initiate a molecular cascade, which involves PI3K/AKT, and eventually activates S6K. However, PA can directly act on TOR and S6K in response to amino acid sensing, or resistance exercise in skeletal muscle, in a PI3K-indepedent fashion (O'Neil et al., 2009; Fang et al., 2001). In the exclusive presence of both acute and chronic ethanol exposure, PLD produces the lipid phosphatidylethanol (PEt),

while PA levels decrease in parallel with the increase in PEt. Acute ethanol intake also decreases PLD activity in the rat pancreas 1 -2 hours after ethanol consumption (Rydzewska et al., 1996).

While PEt degradation is slow compared to its synthesis, accumulation of PEt in various tissues is reflective of both the presence, and amount of ethanol. PEt has been previously found in brains of ethanol treated rats (Lundqvist et al., 1994), and in leukocytes of alcoholics (Hoek et al., 1992; Rodriguez et al., 1996; Alling et al., 1984; Varga et al., 2000). Taken together, if ethanol inhibits PLD activity and suppresses S6K-P in the nervous system, it should be tested whether neuronal S6K-P inhibition by a 20-minute treatment with sedating ethanol doses (Figure 6), is mediated via PLD.

Future Directions

Multiple questions remain unanswered from my doctoral research, and they will be investigated in future studies. To delineate research questions for future directions. First, do Rac1 and Arfip participate with Arf6 to regulate InR signaling, or are they simply mediating a signal to Arf6 from a distinct receptor-signaling pathway? If Rac1 and Arfip regulate InR signaling, do they regulate InR signaling through the actin cytoskeleton or by functioning in the intracellular InR signaling cascade? Can insulin treatment recruit Arf6.GTP? And if so, which guanine nucleotide exchange regulator activates Arf6 upon insulin treatment?

Does lack of Arf6 impair phosphorylation of the actin-severing protein cofilin *in vivo* and cell culture? Does insulin treatment induce cofilin –phosphorylation and actin polymerization in S2 cells? If so, does the lack of Arf6, wortmannin, or rapamycin inhibit insulin-induced cofilin phosphorylation? Does Arf6-Q67L induce cofilin phosphorylation? Are cofilin and S6K phosphorylated by Arf6 in the same cells? Does treatment with F-actin polymerization inhibitor cytochalasin D, inhibit insulin and or Arf6-induced S6K-P? Does insulin induce changes in the actin cytoskeleton using Rhodamine-phalloidin staining as markers for the actin cytoskeleton?

Second, does Arf6 endocytose and/or recycle the InR? If so, is this the prime mechanism by which Arf6 regulates InR signaling or does it play a dual and separable role in InR internalization and InR signaling? If Arf6 does function in InR endocytosis, does it internalize the inR via CDE or CIE? Does Arf6-mediated internalization and/or recycling of InR require trafficking via endosomes positive for Rab GTPases? Does Arf6 function in InR endocytosis through its role in the actin cytoskeleton, or does it have a direct role in InR internalization and/ or recycling? Does treatment with cytochalasin D inhibit Arf6/Insulin-induced InR internalization? What are the actin dynamics in Arf6 Q67L and/or Arf6 RNAi expressing cells alone versus insulin-treated Arf6-Q67L and/or Arf6 RNAi expressing cells at distinct insulin incubation time points?

If Arf6 positively regulates InR endocytosis and signaling, can PLD activation under conditions of Arf6 knock-down rescue both InR internalization and signaling, or can it rescue InR signaling but not internalization? Are Arf6 mutants insulin resistant when previously receiving a glucose challenge, further substantiating that they are indeed impaired in InR signaling? Does insulin resistance induced by a high sucrose or high fat diet increase ethanol preference?

Third, is Arf6 required and sufficient in the mushroom bodies (MBs) to regulate acute ethanol behaviors? If so, can the function of Arf6 be narrowed to a specific subset of MB neurons? Is the InR receptor expressed in these neurons? And if so, is Arf6 downstream of InR signaling in these neuronal subsets? Do these neurons encode neurochemicals that are relevant for alcohol addiction in mammals? RhoGAP18B transcript RC is involved in ethanol sedation, while RA is involved in ethanol hyper-locomotion (Rothenfluh et al., 2006), does RA over-expression in Arf6 mutants fail to reduce ethanol-induced hyper-locomotion, while ethanol sedation resistance by over-expression of RC is suppressed in Arf6 mutants?

Fourth, how does acute ethanol affect InR signaling including neuronal S6K-P? Given that acute ethanol exposure inhibits PLD, is this the main reason why intoxicating ethanol doses inhibit S6K-P? If so, do lower ethanol doses increase PLD and thus increase S6K-P? Why do Arf6 mutants lack neuronal

S6K-P even when exposed to ethanol doses that increase S6K-P? Do Arf6 mutants express less InRs and therefore lack InR signaling, or does lack of Arf6 impair PLD activation and hence the downstream InR signaling cascade? Can acute ethanol impair InR endocytosis as it does InR signaling? A recent paper by Dietz et al (2012) has shown that repeated cocaine exposure in mice downregulates Rac1.GTP, and that this repression impairs cofilin signaling, which contributes to alterations in dendritic spine morphology in NAc neurons. Can other substances like ethanol also regulate neuronal activity of the Arf or Rho family of small GTPases? More specifically, can acute ethanol or repeated ethanol exposure, alter Arf6 or Rac1GTPase activity in the nervous system to mediate ethanol preference via cofilin or S6K-P?

Fifth, social isolation and genetic manipulations in Rheb, an effector of TOR signaling, influence synapse number (Eddison et al., 2011; Knox et al., 2007), and both social isolation and NS over expression of Rheb causes behavioral ethanol sensitivity (Eddison et al., 2011). While Arf6 mutants show no gross morphological defects, do they show ultrastructural defects in synapse number, which may explain why they are so sensitive to ethanol? Can synapse number be rescued by expressing constitutive active S6K in the Arf6 mutant nervous system or MBs, which produces behavioral resistance to ethanol sedation? Given that Arf6 has been implicated in synaptic plasticity, do Arf6

mutants have a defect in synapse size? Can a defect in synaptic plasticity cause behavioral sensitivity to ethanol sedation?

Finally, a future research direction is to study Arf6 and InR signaling in the context of ethanol preference. Is Arf6 involved in ethanol preference? How does naive ethanol sensitivity correlate with ethanol preference and consumption? If Arf6 is involved in ethanol preference, does it function in learning and memory components of ethanol preference? It has been shown that flies require ethanol sedation to display ethanol preference under an ethanol pre-treatment paradigm (Devineni et al., 2009), which suggests that ethanol dosage is a factor for ethanol preference. However, in this protocol the ethanol dosage is delivered in a single exposure/session. What would happen if flies receive the same ethanol dosage but spacing the ethanol delivery by intermediate recovery periods in a massed vs. spaced training paradigm and then tested on a two-choice drinking assay 1-3 plus days after the exposures.

Although spaced training maximizes associative learning (Chen et al., 2012), it would also reduce ethanol sedation. Therefore, is ethanol sedation required for ethanol preference? Which ethanol exposure paradigm produces a long lasting form of ethanol preference? Does 24 hours ethanol preference require protein synthesis, or is it only 3 days ethanol preference that requires it? If mass training produces the strongest form of ethanol preference, would flies delivered ethanol under this paradigm overcome an aversive stimulus (i.e.

electric shock or the bitter taste of quinine) to drink ethanol? Which neurons are involved in compulsive drinking-like behavior?

If Arf6 mutants are impaired at ethanol preference, is the neuronal loci required to rescue Arf6's ethanol sensitivity also required to rescue ethanol preference? Is InR signaling required for ethanol preference and is it via positive regulation? Can S6K-over expression in Arf6 mutants rescue ethanol preference? It is known that quinine induces taste aversion, however, ethanol-preferring flies overcome quinine's bitter taste to consume ethanol-containing food. This suggests the existence of a regulatory "switch" in taste recognition that can initially control consumption, but that is turned "off" during the establishment of ethanol self-administration behavior. Is this 'switch' encoded in neurons involved in taste? Which molecules are at the interface of taste recognition and ethanol preference behavior? Is this switch irreversibly turned "off" in ethanol preferring flies? If not, can neuronal and/or genetic activation of this "molecular switch" reverse ethanol preference behavior? Are there "molecular switches" in other sensory modalities that contribute to the development of ethanol addiction?

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