Title
Molecular substrates and biochemical pathways regulating acute ethanol sensitivity in the fruit fly, Drosophila melanogaster

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Molecular Substrates and Biochemical Pathways Regulating Acute Ethanol Sensitivity in the Fruit Fly, Drosophila melanogaster

by

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DISSERTATION

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In loving memory of my mother, Chiu-lian J. Corl

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San Francisco, October 19h, 2007

To whom it concerns:

I hereby state that the work carried out by Ammon B. Corl for the purpose of his Ph.D. requirement is comparable to theses or dissertations normally awarded by the UCSF Graduate Program in Neuroscience. The work carried out in the screen as described in Chapter 2 was carried out by Ammon Corl and Aylin Rodan. The work described in Chapter 3, which has been submitted for publication, was carried out by Ammon Corl in its majority; the quantitative PCR experiments were performed by Karen Berger and Julie Gesch. The work described in Chapter 4, which is published in the journal Nature Neuroscience, was carried out by Ammon Corl in its majority; the initial experiments which inspired the paper were carried out by Aylin Rodan.

Sincerely,

Ulrike Heberlein
Professor

Molecular substrates and biochemical pathways regulating acute ethanol sensitivity in the fruit fly, *Drosophila melanogaster*

Ammon B. Corl

Abstract

Our knowledge concerning the mechanisms through which ethanol acts on the central nervous system to elicit behavioral changes is still far from complete. In recent years, the fruit fly, *Drosophila melanogaster*, has proven itself as an attractive model system in which to study the genes and pathways that modify acute and chronic behavioral responses to ethanol exposure.

We conducted a genetic screen in *Drosophila* using a locomotor video tracking system to identify mutants with altered ethanol sensitivity. Out of a total of 1483 lines screened, 21 mutants were isolated and selected for further characterization. These mutant lines were subjected to a battery of genetic, behavioral, and molecular tests, including back-crossing to the parental strain, locomotor tracking at various ethanol doses, and analyses for central nervous system GAL4 expression, ethanol absorption, negative geotaxis, and inverse PCR. These results are discussed in Chapter 2.

From the forward genetic screen described above, we identified a mutant, *happyhour* (*hppy*), that is resistant to the sedative effects of ethanol. While loss-of-function mutations in *hppy* resulted in resistance to ethanol-induced sedation, neuronal overexpression of *hppy* caused increased sensitivity. Although *hppy* shows strong

homology to mammalian Ste20 family kinases involved in JNK signaling, we found that neither activation nor inhibition of the JNK pathway affected ethanol-induced sedation. Interestingly, perturbations of a second MAP kinase pathway, the EGFR/ERK pathway, in neuronal tissues strongly affected sensitivity to ethanol-induced sedation. Genetic interaction experiments between *hppy* and the EGFR/ERK pathway suggest a role for *hppy* as an inhibitor of the pathway, functioning downstream of the EGFR but upstream of ERK. These results are discussed in Chapter 3.

In a study to better understand the neuroanatomical substrates and molecular pathways underlying ethanol sensitivity in *Drosophila*, we found that genetic manipulation of a small group of insulin producing cells results in a strong increase in sensitivity to ethanol-induced sedation. In addition, various mutations that impair the function of the insulin receptor (InR) signaling pathway, as well as transgenic flies that have disrupted neuronal InR signaling, also have increased sensitivity to the intoxicating effects of ethanol. These results are discussed in Chapter 4.

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Chapter 1: Introduction

Introduction

Alcohol.

Certainly never in history has there been a drug more praised:

"If I die I must, let me die drinking in an inn."

-Walter Map, medieval writer (c.1140 – c.1209)

"Sometimes too much drink is barely enough."

-Mark Twain, author (1835 – 1910)

or more vilified:

"O thou invisible spirit of wine, if thou hast no name to be known by, let us call thee devil."

-William Shakespeare, playwright (1564-1616)

"Drinking makes such fools of people, and people are such fools to begin with, that it's compounding a felony."

-Robert Charles Benchley, writer (1889-1945)

Alcohol (ethanol: C_2H_5OH) is one of the oldest drugs of abuse used by humankind: pottery wine vessels dating back more than 5,000 years were discovered in the Shandong province of China (Du and Du, 2002). It remains one of the most popular and widely consumed drugs to this day: in the United States, 73% of adults consume alcohol (2001). While the pleasurable and disinhibiting effects of alcohol consumption are enjoyed by many of us, for some people alcohol consumption leads to alcohol addiction, a devastating illness with enormous medical and societal costs. In the United States, for

example, approximately 7% of adults are alcoholics, and alcohol-related problems cost the country approximately $176 billion per year, and are responsible for 100,000 deaths (Diamond and Gordon, 1997; Volpicelli, 2001). A better understanding of the genetic and environmental factors that contribute to the development of alcoholism would provide considerable benefits to those who suffer from alcohol addiction and to society in general.

Although the cognitive and behavioral changes associated with alcohol consumption are quite familiar to most adults, our knowledge concerning the mechanisms through which ethanol acts on the central nervous system to produce these behavioral changes is still far from complete. Decades of *in vitro* and *in vivo* studies in mammalian and invertebrate systems have shown that rather than acting on a single molecular target, ethanol exerts effects on multiple different voltage-gated and ligand-gated ion channels, and its actions are transduced through a variety of intracellular signaling cascades (reviewed in Diamond and Gordon, 1997; Crabbe et al., 2006).

In the past decade, the fruit fly, *Drosophila melanogaster*, with its accessibility to genetic and molecular analysis, has proven itself as an attractive model system in which to study the genes and pathways that modify acute and chronic behavioral responses to alcohol. When exposed to ethanol vapors, fruit flies behave similarly to mammals: low doses of ethanol result in hyperactivity, whereas higher doses result in decreased activity and eventual loss of postural control and sedation (Wolf and Heberlein, 2003). Recent studies using *Drosophila* have revealed much about the various molecules and biochemical pathways that underlie the ethanol behavioral response. Several of these molecules and pathways have been found to have similar roles in mediating ethanol behaviors in mammals, validating the usefulness of *Drosophila* as a valuable tool for identifying candidate genes and pathways underlying the behavioral response to ethanol.

In this dissertation, I will describe results from my research in which I have employed *Drosophila* as a model organism to identify novel genes and pathways that

regulate the behavioral response to ethanol. I will first, in Part 1 of the introduction, review the current literature regarding what molecules and pathways are already known to regulate the ethanol response, with particular emphasis on transgenic and knockout studies in mammalian systems. In Part 2 of the introduction, I describe in detail the current literature regarding the use of *Drosophila* as a model organism for studying the behavioral effects of drugs of abuse, with particular emphasis on ethanol and cocaine studies. I conclude the introduction with Part 3, in which I describe two biochemical pathways which I have found to play a role in mediating the ethanol response in *Drosophila*: the insulin / insulin-like growth factor signaling (IIS) pathway and the epidermal growth factor receptor / extracellular signal-regulated protein kinase (EGFR / ERK) cascade. In each case I will describe the signaling pathway itself and then review specific aspects of the literature for each pathway, with special emphasis given to examples of neuronal roles of the IIS and EGFR / ERK pathways.

I have divided the main body of my dissertation into three chapters. In Chapter 2, I describe a forward genetic screen I conducted in order to identify novel molecules regulating the behavioral responses to ethanol in *Drosophila*, and describe the various mutants I isolated from that screen. In Chapter 3, I present detailed results for one particular mutant isolated from this screen, a mutant which I have named *happyhour*, and describe how this mutant led me to investigate a role for the EGFR / ERK pathway in regulating ethanol-induced sedation. In Chapter 4, I describe the results from my study of another ancient and conserved pathway, the IIS pathway, and describe how perturbation of this pathway also results in the disruption of normal ethanol sensitivity in *Drosophila*. Finally, I will draw conclusions from my collective data and present possible future directions for further studies in this exciting area of research.

Part I: Alcohol Studies in Mammalian Systems

Alcohol Studies in Humans

Alcohol dependence (alcoholism), defined by the Journal of the American Medical Association as " a primary, chronic disease characterized by impaired control over drinking, preoccupation with the drug alcohol, use of alcohol despite adverse consequences, and distortions in thinking" (1992), is a devastating illness that affects approximately 7% of adults in the United States (Diamond and Gordon, 1997). Alcohol-related disorders are responsible for greater than 105,000 annual deaths in the United States alone (McGinnis and Foege, 1999), a country where greater than 20% of all hospitalized patients have a medical disorder related to heavy drinking (Diamond and Gordon, 1997). Alcohol dependence, ranking with mood and anxiety disorders among the top three psychiatric disorders in the United States (Kessler et al., 1994), incurs a staggeringly high societal cost of approximately $176 billion dollars per year (Volpicelli, 2001). For all these reasons, research aimed at trying to identify the genetic and environmental factors that underlie alcoholism is certainly well merited.

There is strong evidence for a genetic component to alcoholism. The heritability of risk for alcohol dependence varies from approximately 39%, estimated from adoption studies (Cloniger et al., 1981), to approximately 60%, estimated from twin studies (Heath et al., 1997). Despite this knowledge of inherited contributions to alcoholism, thus far only two genes have been conclusively identified that affect alcoholism risk. Variants in these genes, encoding the metabolic enzymes aldehyde dehydrogenase and alcohol dehydrogenase, exert protective effects against the development of alcoholism (Enoch

and Goldman, 2001). Interestingly, multiple studies in humans have shown a correlation between acute ethanol sensitivity and alcoholism, indicating that the level of response to intoxicating doses of ethanol can act as a predictor of future alcoholism (Schuckit et al., 2004). For example, a low level of response to ethanol at age 20 was found to be associated with a four-fold increased likelihood of development of alcoholism within the following ten years (Schuckit, 1994; Schuckit and Smith, 1996). This strongly suggests that the identification of genes and pathways mediating acute responses to ethanol promises to offer helpful insight into the genetic factors contributing to alcohol addiction.

In an effort to identify chromosomal regions and/or specific genetic loci that are associated with alcohol dependence in humans, various linkage studies have been performed, with limited success. A whole-autosomal genome scan for genetic linkage to alcohol dependence performed on members of a Southwestern American Indian tribe revealed evidence of linkage on chromosome 11p, in close proximity to the *DRD4* dopamine receptor and tyrosine hydroxylase (*TH*) genes, as well as linkage on chromosome 4p, near the $GABA_A$ β1 receptor subunit gene (Long et al., 1998). In addition, other genome-wide searches for alcoholism susceptibility loci have suggested evidence for linkage on chromosomes 1, 2, 3, and 7 (Reich et al., 1998; Foroud et al., 2000). As *in vitro* electrophysiological studies and *in vivo* studies of knockout and transgenic mice have strongly suggested a role for the $GABA_A$ receptor in mediating ethanol-induced behaviors (see below), studies have been done to determine whether linkage associated with alcohol dependence could be established in or near $GABA_A$ receptor subunit gene regions of the human genome. Two different linkage studies involving large numbers of alcohol dependent subjects both found that variation in the

$GABA_A$ α2 receptor subunit gene (*GABRA2*) was strongly associated with alcohol dependence (Edenberg et al., 2004; Lappalainen et al., 2005). In a separate linkage study of Finnish and Native American male and female subjects, evidence for an alcohol dependence locus in the $GABA_A$ gene cluster region on chromosome 5, particularly in the $GABA_A$ α6 receptor subtype gene region (*GABRA6*), was reported (Radel et al., 2005). Lastly, a study of alcohol-dependent adults found that specific polymorphisms of the corticotropin releasing hormone receptor 1 (*CRHR1*) gene were associated with increased ethanol intake in ethanol-dependent subjects (Treutlein et al., 2006). A role for CRHR1 in mediating ethanol consumption behavior was not too surprising, as previous research in mice had found that knockout mice deficient for corticotropin-releasing hormone receptors (*Crhr1* -/-) were found to display enhanced and progressively increased ethanol-intake behaviors in response to stress (Sillaber et al., 2002).

Alcohol studies in Rodents

Due to the complexity of performing alcohol studies on human subjects, researchers have turned to other animals to use as model organisms in alcohol research. Rodents display similar behavioral responses to alcohol as humans: lower concentrations of ethanol induce locomotor stimulation, whereas higher ethanol concentrations induce locomotor incoordination, ataxia, sedation, and death (Frye and Breese, 1981; Smoothy and Berry, 1985). Researchers have developed a plethora of assays to quantitatively measure the various behavioral effects of ethanol in rodents (Crabbe et al., 2006). Ethanol-induced increases in locomotor activity are often quantified by measuring movement in an open field assay, whereas the sedative/hypnotic effects of ethanol are

measured in a loss-of-righting reflex (LORR) assay, where duration of and latency to ethanol-induced sleep are measured. Ethanol-induced incoordination and ataxia can be measured using a variety of assays that test the animal's locomotor coordination: these include the rotarod, stationary dowel, and grid tests. The anxiolytic effects of ethanol can be quantified in an elevated plus maze, in which the times spent in the open versus the closed arms of the maze are measured and compared. Rodents develop functional tolerance to ethanol – this can be measured using the above-mentioned assays after the animals have been subjected to repeated ethanol administrations. Rodents can develop preference for ethanol, which can be measured using several assays, including a two-bottle choice assay where rodents can choose to drink from an ethanol-solution containing bottle or a water containing bottle *ad libitum*, a conditioned place preference assay where rodents learn to associate an environment with a positive ethanol stimulus, or operant models where rodents press a lever to receive an ethanol reward. Rodents can also develop physical dependence on ethanol, and severity of withdrawal symptoms can be quantified by measuring handling-induced convulsions. Various methods have been proposed to model alcoholism in rodents. These models, including the reinstatement model, the alcohol deprivation model, and the point-of-no-return model, attempt to model specific aspects of alcohol addiction seen in humans including craving, relapse, and loss of control over drinking (Spanagel, 2000).

The ability to target and inactivate specific genes through homologous recombination (commonly known as generating a gene "knockout") or overexpress a gene of interest in transgenic mice has proven to be extremely useful for researchers hoping to use mice to better understand the pathways that underlie ethanol-induced

behavior and ethanol addiction in mammals (Palmiter et al., 1982). Many potential targets of ethanol action (identified through *in vitro* electrophysiological studies, QTL studies, and pharmacological studies involving quantifying ethanol-induced effects after injection of receptor agonists or antagonists) have been "knocked-out" or overexpressed in transgenic mice. The effects of such genetic manipulations on ethanol-induced behaviors have been measured using the various assays described above and will be described in detail later in this section. It is important to keep in mind that genetic background can profoundly affect the phenotypes observed: a null mutation in a particular gene that causes a certain ethanol-induced phenotype in one background strain may not reveal the same phenotype when tested in another background strain (Crabbe et al., 2006). In addition, one must always consider that the nervous system is plastic and that compensatory changes may occur during the development of a knockout or transgenic mouse that may mask the true contribution of a particular protein of interest in transducing ethanol's many effects. In this section of the introduction, I will describe in detail research done using rodent models to identify receptors, channels, and signaling pathways that mediate the behavioral effects of ethanol, with a particular emphasis on studies using knockout and transgenic mice. While studies have been done in mice to identify ethanol-related genes through QTL analysis and genome-wide mutagenesis utilizing forward genetic screens (Belknap et al., 2001), the insight that these approaches have given us into the field of ethanol research has unfortunately been sparsely limited and thus I have chosen not to focus on them in my introduction.

A.) GABA / $GABA_A$ Receptors

Ionotropic γ-aminobutyric acid type A ($GABA_A$) receptors are the major inhibitory neurotransmitter receptors in the mammalian brain, conducting chloride ions through their channel pores upon ligand binding. $GABA_A$ receptors are pentameric and, while they can be composed of a number of different subunits (taken from the α, β, γ, δ, ε, and π classes), most native $GABA_A$ receptors are thought to contain α, β, and γ subunits (McKernan and Whiting, 1996). It has been known for quite some time that ethanol potentiates $GABA_A$ receptor-induced chloride currents in a number of preparations, including chick spinal cord neurons and *Xenopus* oocytes (Celentano et al., 1988; Waffard et al., 1990), and specific amino-acid residues in transmembrane domains 2 and 3, conserved across various $GABA_A$ receptor subunits, have been identified as being critical for the allosteric modulation of $GABA_A$ receptors by alcohol (Mihic et al., 1997).

Pharmacological studies have suggested a role for $GABA_A$ receptors in modulating behavioral responses to ethanol (reviewed in Mihic, 1999). For example, administration of the $GABA_A$ receptor agonist muscimol increases the duration of ethanol-induced LORR, while administration of the $GABA_A$ antagonist bicuculline has the opposite effect (Liljequist and Engel, 1982; Martz et al., 1983). In addition, there is pharmacological evidence that the $GABA_A$ receptor may regulate ethanol drinking, as injection of bicuculline into the ventral tegmental area, a region of the midbrain involved in mediating reward-related behaviors, results in a decrease in ethanol consumption (Nowak et al., 1998).

In the past decade, quite a few studies have been published describing various ethanol-induced phenotypes observed in knockout mice deficient for the various $GABA_A$ receptor subunits. For example, mice lacking the $GABA_A$ receptor α1 subunit (α1 -/-) were generated and tested in a variety of ethanol behavioral assays. While α1 -/- mice show decreased ethanol consumption and preference in a two-bottle choice assay (Blednov et al., 2003a; June et al., 2007), increased ethanol-induced locomotor stimulation in an open field assay (Blednov et al., 2003a; Kralic et al., 2003; June et al., 2007), decreased ethanol operant self-administration in a lever pressing assay (June et al., 2007), and increased ethanol-induced conditioned taste aversion (Blednov et al., 2003a), they are normal for other behaviors, including ethanol-induced ataxia on the rotarod (Kralic et al., 2003), ethanol-induced anxiolytic behaviors in an elevated plus maze (Kralic et al., 2003), acute functional ethanol tolerance as measured on a stationary dowel (Kralic et al., 2003), ethanol-induced conditioned place preference (Blednov et al., 2003a), and severity of handling-induced convulsions during ethanol withdrawal (Blednov et al., 2003a). One study found that male α1 -/- mice are more sensitive to the sedative/hypnotic effects of ethanol as measured in a LORR assay (Blednov et al., 2003b). This was not observed, however, in a second study (Kralic et al., 2003).

Knockout mice have also been generated for the α2, α5, and α6 subunits of the $GABA_A$ receptor. α2 deficient mice were shown to have decreased preference for ethanol consumption in the two-bottle choice assay (in females only) and decreased duration of sleep time during the LORR assay, but have normal levels of ethanol-induced anxiolytic behaviors in the elevated plus maze and normal levels of handling-induced convulsions during ethanol withdrawal (Boehm et al., 2004a). α5 knockout mice display decreased

ethanol consumption and preference in the two-bottle choice assay (Boehm et al., 2004a; Stephens et al., 2005) and decreased severity of handling-induced convulsions during ethanol withdrawal (Boehm et al., 2004a), but show wild-type levels of sensitivity to ethanol-induced ataxia on the rotarod (Stephens et al., 2005), ethanol-induced LORR (Boehm et al., 2004a; Stephens et al., 2005), ethanol-induced anxiolytic behaviors in the elevated plus maze (Boehm et al., 2004a), and ethanol operant self-administration in a lever pressing assay (Stephens et al., 2005). In addition, α6 deficient mice have been generated and studied. While α6 -/- mice behave normally for a wide variety of ethanol-induced behaviors, including ataxia (Korpi et al., 1998), LORR (Homanics et al., 1997), acute and protracted ethanol tolerance (Homanics et al., 1998), and handling-induced convulsions during ethanol withdrawal (Homanics et al., 1998), α6 -/- mice do show decreased ethanol-induced locomotor activation in a stair-climbing assay (Korpi et al., 1998).

Null mutants in the β subunits of the $GABA_A$ receptor have also been made, specifically for the β2 and the β3 subunits. β2 knockout mice show several ethanol phenotypes, including reduced sensitivity to ethanol-induced sedation in the LORR assay (in males only) (Blednov et al., 2003b), decreased ethanol-induced conditioned place preference (Blednov et al., 2003a), and increased severity of handling-induced seizures during ethanol withdrawal (Blednov et al., 2003a). β2 -/- mice show wild-type behavior in several other assays, including those measuring ethanol consumption, ethanol-induced locomotor activity, and ethanol-induced conditioned taste aversion (Blednov et al., 2003a). Mice deficient in the β3 subunit were tested for their sensitivity to the

sedative/hypnotic effects of ethanol - they display no differences from wild-type mice in their sleep times in the LORR assay (Quinlan et al., 1998).

The $GABA_A$ receptor γ2 subunit has two splice variants: a long form, γ2L, and a short form, γ2S. Transgenic mice overexpressing either the long or the short splice form of the γ2 subunit of the $GABA_A$ receptor show decreased acute functional tolerance to ethanol as measured on a stationary dowel, but show normal behaviors when tested for ethanol-induced LORR, ethanol-induced ataxia on the stationary dowel, and handling-induced convulsions during ethanol withdrawal (Wick et al., 2000). As homozygous γ2 knockout mice die within a few days of birth (Gunther et al., 1995), γ2 -/- mice (obviously) could not be tested for their ethanol-induced behaviors. Knockout mice bearing a deletion specifically affecting the long splice variant of the γ2 subunit have been generated and appear healthy: these γ2L -/- mice show wild-type phenotypes for a variety of ethanol-induced behaviors, including ethanol-induced LORR, ethanol-induced anxiolytic behavior in an elevated plus maze, acute functional tolerance to ethanol, ethanol-stimulated locomotor activity, and handling-induced convulsions during ethanol withdrawal (Homanics et al., 1999), suggesting that the long splice variant of the $GABA_A$ γ2 subunit (γ2L) is not involved in mediating the acute behavioral responses to ethanol in mice.

Lastly, the $GABA_A$ receptor δ subunit has also been knocked out in mice, and the consequences of its removal have been studied by industrious ethanol researchers. Interestingly, δ subunit knockout mice (δ -/-) have decreased ethanol consumption and preference in a two-bottle choice assay and decreased severity of handling-induced convulsions during ethanol withdrawal (Mihalek et al., 2001). δ -/- mice show normal

levels of ethanol-induced ataxia on the rotarod, normal sensitivity to ethanol-induced sedation in the LORR assay, normal ethanol-induced anxiolytic behavior in the elevated plus maze, normal chronic and acute ethanol tolerance, and normal levels of ethanol-induced hypothermia (Mihalek et al., 2001). $GABA_A$ receptors containing the δ subunit are thought to be localized primarily outside of the synapse (Hanchar et al., 2004), raising the intriguing possibility that ethanol may mediate its behavioral effects, at least in part, through its actions on extrasynaptically localized neurotransmitter receptors.

In addition to studying mice deficient for the various $GABA_A$ receptor subunits, the effects of perturbing the function of the GABA transporter subtype 1 (GAT1), the major GABA transporter responsible for mediating the rapid reuptake of synaptically released GABA, has also been studied. While GAT1 deficient mice (GAT1 -/-) show normal levels of ethanol consumption, they display several alterations in ethanol-induced behaviors, including decreased sensitivity to the sedative/hypnotic effects of ethanol in the LORR assay, decreased ethanol-induced conditioned place preference, decreased ethanol-induced conditioned taste aversion, and decreased ethanol-induced locomotor stimulation (Cai et al., 2006). Other manipulations of GAT1 activity have yielded results that conflict with the LORR results observed in GAT1 -/- animals, however, as it was found that injection of the GAT1 antagonists ethyl nipecotate or NO-711 results in increased, rather than decreased, ethanol-induced sedation in the LORR assay (Hu et al., 2004). Furthermore, transgenic mice overexpressing GAT1 show decreased ethanol-induced sleep time in the LORR assay (Hu et al., 2004). The reasons underlying these apparent discrepancies are not clear.

While in recent years we have certainly seen a prodigious number of studies examining the ethanol-induced behavioral effects of mice lacking various $GABA_A$ receptor subunits, there are many questions left unanswered. It has been proposed, for example, that lack of apparent ethanol phenotypes in various $GABA_A$ receptor subunit knockout mice may be due to compensatory substitution of other subunits for the ones that have been knocked out (Crabbe et al., 2006); this problem could perhaps be avoided by utilizing knock-in mice where a mutated subunit is substituted in place of the wild-type subunit. In addition, the consequences of mutations in glutamic acid decarboxylase, the principle enzyme responsible for GABA synthesis, or mutations in metabotropic $GABA_B$ receptor subunits have yet to be explored. A potential role for the activity of presynaptic $GABA_B$ receptors in reducing ethanol potentiation of GABAergic synaptic transmission has been described (Ariwodola and Weiner, 2004), suggesting the intriguing possibility that $GABA_B$ receptors may perhaps play a role in mediating behavioral sensitivity to ethanol.

B.) Glutamate / Glutamate Receptors / Fyn Kinase

As acute exposure to pharmacologically relevant concentrations of ethanol has been shown to inhibit ion current through both NMDA and AMPA type ionotropic glutamate receptors (Lovinger et al., 1989; Mameli et al., 2005), several behavioral studies have been conducted in transgenic mice to explore the roles that these and other glutamatergic systems might play in regulating ethanol-induced behaviors.

N-methyl-D-aspartate (NMDA) receptors, known for their roles in mediating synaptic plasticity, are ionotropic glutamate receptors composed of an NR1 and at least

one type of NR2 subunit (Cull-Candy et al., 2001). Knockout mice lacking the NMDA receptor NR2A subunit (NR2A -/-) have been generated and tested for a battery of ethanol phenotypes. Interestingly, while NR2A -/- mice are normal in a variety of ethanol assays, including ethanol-induced locomotor activation in an open field assay, ethanol-induced sleep time in a LORR assay, ethanol consumption in a two-bottle choice assay, and ethanol-induced hypothermia, they do show increased ethanol-induced locomotor incoordination on the accelerating rotarod, as well as strongly reduced ethanol-induced conditioned place preference (Boyce-Rustay and Holmes, 2006). Knockout mice lacking the NMDA ε1 subunit have also been tested in a LORR assay: while they show normal levels of acute sensitivity to the sedative/hypnotic effects of ethanol, they show impaired development of tolerance to repeated ethanol stimuli as measured in the LORR assay (Sato et al., 2006). Pharmacological administration through intraperitoneal (i.p.) injection of a selective antagonist of the NMDA NR2B subunit, Ro 25-6981, was found to potentiate the sedative/hypnotic effects of ethanol in wild-type mice, suggesting a role for the NR2B subunit in mediating the intoxicating effects of ethanol (Boyce-Rustay and Holmes, 2005).

Indirect evidence for the involvement of NMDA receptor signaling in regulating ethanol-induced behaviors has come from studies of knockout mice lacking Fyn kinase (Fyn -/-), a Src family non-receptor tyrosine kinase which has been shown to enhance NMDA receptor channel function through tyrosine phosphorylation of the long intracellular tail of the NMDA receptor NR2B subunit (Yaka et al., 2002; Yaka et al., 2003a). Fyn -/- mice display increased sleep time in the LORR assay (Miyakawa et al., 1997; Boehm et al., 2003; Yaka et al., 2003b). Importantly, this difference between Fyn -

/- and Fyn +/+ mice in the LORR assay is abolished through systemic injection of the NMDA selective antagonist ifenprodil (Yaka et al., 2003b). While Fyn -/- mice show normal levels of ethanol-induced conditioned place preference (Yaka et al., 2003b) and ethanol-induced hypothermia (Boehm et al., 2003), they have increased sensitivity to the anxiolytic effects of ethanol in the elevated plus maze (Boehm et al., 2003) and impaired acute tolerance to the incoordinating effects of ethanol when measured on the stationary dowel, but not the rotarod (Boehm et al., 2003). While one study found that Fyn -/- male mice show decreased ethanol preference in a two-bottle assay (Boehm et al., 2003), another study did not find a difference between Fyn deficient mice and controls in this assay (Yaka et al., 2003b). Mice overexpressing Fyn kinase in the forebrain have also been developed and studied: they were found to have decreased sensitivity to the sedative/hypnotic effects of ethanol in the LORR assay and reduced ethanol preference in the two-bottle choice assay (Boehm et al., 2004b).

Mice deficient for several other proteins that are tangentially involved in NMDA receptor signaling have also been shown to display ethanol phenotypes. For example, mice deficient for Eps8, a regulator of actin dynamics that is part of the NMDA receptor complex, have abnormally increased NMDA receptor currents (Offenhauser et al., 2006). When compared to controls, Eps8 knockout mice consume more ethanol in the two-bottle choice assay, are resistant to the intoxicating effects of ethanol as measured on the rotarod and the LORR assay, and show slightly increased ethanol-induced locomotor activation in the open field assay (Offenhauser et al., 2006). Mice deficient in another protein believed to be involved in the anchoring and synaptic localization of NMDA receptors, Homer2, were also studied and found to have a variety of ethanol phenotypes,

including decreased ethanol consumption of a relatively high (12%) concentration of ethanol in the two-bottle choice assay, increased sleep time in the LORR assay, and decreased ethanol-induced locomotor sensitization and conditioned place preference (Szumlinski et al., 2005). Finally, *Per2*^{Brdm1} mutant mice, which bear a mutation in the circadian clock gene *Period2* and have increased glutamate levels in the extracellular space of the brain due to decreased expression levels of the glutamate transporter *Eaat1*, were also found to have ethanol phenotypes. Specifically, *Per2*^{Brdm1} mice show increased ethanol intake in the two-bottle choice assay and increased operant self-administration of ethanol in a lever pressing assay (Spanagel et al., 2005).

Outside of the NMDA receptor, the literature regarding roles for other glutamate receptors in regulating ethanol-induced behaviors is rather sparse. Mice deficient for the AMPA GluR1 subunit have decreased sensitivity to ethanol-induced hypothermia, but are normal for a wide variety of ethanol behaviors, including LORR, ataxia on the rotarod, ethanol tolerance, ethanol consumption in the two-bottle choice assay, ethanol-induced locomotor activity, and the expression of an alcohol deprivation effect (ADE), which is used as model of relapse-like drinking behavior (Cowen et al., 2003). In another study, in which AMPA receptor signaling was perturbed either pharmacologically using the AMPA antagonist GYKI 52466 or by knocking out the AMPA receptor GluR-C subunit, it was found that while neither perturbation causes a significant change in ethanol consumption in the two-bottle choice assay, either perturbation is sufficient to reduce the expression of the alcohol deprivation effect and to reduce cue-induced reinstatement, a model for ethanol seeking behavior, suggesting a role for AMPA receptors in mediating alcohol seeking and relapse behaviors (Sanchis-Segura et al., 2006). Finally, a study done

on null mutant mice lacking the metabotropic glutamate receptor 4 (mGluR4 -/-) found that these mice show decreased ethanol-stimulated locomotor activity, although they show normal levels of ethanol preference in the two-bottle choice assay, normal sensitivity to the sedative/hypnotic effects of ethanol in the LORR assay, and normal levels of ethanol-induced acute withdrawal (Blednov et al., 2004a).

C.) Other Ion Channels

In addition to its enhancement of $GABA_A$ receptor function (see above), ethanol has also been shown to enhance the function of glycine receptors in *Xenopus* oocytes (Mascia et al., 1996). Glycine receptors (Gly-R) constitute one of the major inhibitory neurotransmitter systems in the CNS, localized primarily in the spinal cord and the brainstem where they conduct chloride ions and inhibit neurotransmission (Mihic, 1999). Mutation of a particular serine residue of the Gly-R α-1 subunit, serine 267, is sufficient to abolish the potentiating effects of ethanol on glycine receptors when tested in *Xenopus* oocytes (Mihic et al., 1997). Accordingly, transgenic mice expressing a mutant Gly-R α-1 subunit, mutated at that serine 267, show decreased ethanol sensitivity as measured in several behavioral assays: the rotarod, the LORR assay, and an assay measuring ethanol inhibition of strychnine seizures (Findlay et al., 2002).

Another target of ethanol research has been the neuronal nicotinic acetylcholine receptor (nAChR), specifically the α7 nicotinic receptor subunit. Researchers were interested in testing the *in vivo* roles of the α7 nAChR subunit in mediating ethanol behaviors in α7 knockout (α7 -/-) mice, as it was found *in vitro* that ethanol inhibits agonist activation of α7 homomeric receptors expressed in *Xenopus* oocytes (Yu et al.,

1996), and knockout of the α7 nAChR gene selectively enhances ethanol-induced neurotoxicity in neuronal cortical cultures (de Fiebre and de Fiebre, 2005). These α7 deficient mice were found to display increased ethanol-induced locomotor behavior in an open field test, increased ethanol-induced hypothermia, and increased duration of sleep time in the LORR assay (Bowers et al., 2005).

Potassium channels have also been the targets of considerable study by ethanol researchers. G-protein-activated inwardly rectifying potassium channels (GIRKs) are activated by a variety of upstream agents, including G-protein-coupled neurotransmitter receptors, hormones, and kinases (Dascal, 1997). In *Xenopus* oocytes, it was found that ethanol immediately and reversibly opens GIRK channels (Kobayashi et al., 1999), leading researchers to study the *in vivo* roles of GIRK channels on ethanol-induced behaviors. *Weaver* mutant mice, bearing a missense mutation in the GIRK2 channel, were found to have decreased ethanol-induced analgesia as measured in the tail-flick and hot-plate tests (Kobayashi et al., 1999). Subsequently, knockout mice deficient for GIRK2 (GIRK2 -/-) were generated and tested in a battery of ethanol behavioral assays, where they were found to display decreased ethanol-induced analgesia in the hot plate test (Blednov et al., 2003c), decreased ethanol-induced anxiolytic behaviors in an elevated-plus maze test (Blednov et al., 2001), decreased handling-induced convulsions after acute ethanol injection (Blednov et al., 2001), increased ethanol-stimulated locomotor activity in an open field assay (Blednov et al., 2001), decreased ethanol-induced conditioned taste aversion (Hill et al., 2003), and decreased ethanol-induced conditioned place preference (Hill et al., 2003). No differences were found between GIRK2 -/- and wild-type controls for ethanol consumption as measured in a two-bottle

choice test, for ethanol-induced sleep time in a LORR assay, or for acute functional ethanol tolerance as measured on a rotarod (Blednov et al., 2001).

Studies of other potassium channels have also yielded intriguing results. In *C. elegans*, loss-of-function *slo-1* mutants, which bear mutations in the calcium-activated large conductance potassium channel, are strongly resistant to ethanol-induced reductions in speed of locomotion and frequency of egg laying (Davies et al., 2003). Conversely, gain-of-function mutants in *slo-1* show the opposite phenotypes, i.e., decreased rates of locomotion and egg laying (Davies et al., 2003). In addition, double mutant knockout mice deficient in another type of potassium channel, the Shaw-like voltage-gated potassium channels Kv3.1 and Kv3.3, also display an ethanol behavioral phenotype, showing an increased number of sideways falls after ethanol injection when measured in an open field assay (Espinosa et al., 2001).

Finally, another voltage-gated ion channel, the N-type calcium channel, has also been shown to mediate a variety ethanol-induced behaviors. In studies of knockout mice lacking the N-type calcium channel, it was found that these mice exhibit decreased ethanol consumption in the two-bottle choice assay, decreased sensitivity to the sedative/hypnotic effects of ethanol in the LORR assay, decreased ethanol conditioned place preference, decreased ethanol conditioned taste aversion, and mildly increased locomotor ataxia on the rotarod (Newton et al., 2004).

D.) Dopamine / Dopamine Receptors

One simply cannot review the literature concerning the mechanisms of action of a drug of abuse such as ethanol without discussing the contributions of dopaminergic

systems. Dopamine (DA), signaling through type 1 (D1 and D5) and type 2 (D2, D3, and D4) G-protein coupled dopamine receptors, has been shown to regulate a variety of functions including locomotor behaviors and reward-related behaviors through its activation of mesolimbic and nigrostriatal systems. In studying the rewarding and reinforcing properties of various drugs of abuse, much research has been focused on the mesocorticolimbic pathway, which has as its core component the projection of dopaminergic neurons from the ventral tegmental area (VTA) of the midbrain to the nucleus accumbens (Koob et al., 1992; Wise, 1996). These dopaminergic neurons are thought to be involved in mediating some of the positive, reinforcing properties of various drugs of abuse including cocaine, alcohol, and opiates (Di Chiara, 1995; White, 1996). Studies have found that i.p. injections of various doses of ethanol result in stimulation of DA release specifically in the nucleus accumbens in freely moving rats (Imperato and Di Chiara, 1986), leading diligent scientists to study the effects of knocking out various dopamine receptors and dopamine transporters on ethanol-induced behaviors.

The role for the dopamine D1 receptor subtype was examined in D1 knockout mice (D1 -/-) and in wild-type mice injected with the D1 receptor antagonist SCH-23390. In both cases, compromising D1 receptor function resulted in decreased ethanol consumption and preference as measured in the two-bottle choice assay (El-Ghundi et al., 1998).

Several studies have tackled the role of the dopamine D2 receptor subtype in mediating ethanol behaviors. Mice deficient in the D2 receptor gene (D2 -/-) show decreased ethanol consumption and preference in the two-bottle choice assay (Phillips et

al., 1998; Palmer et al., 2003), a phenotype that is also observed by injecting wild-type mice with the D2 receptor antagonist sulpiride (El-Ghundi et al., 1998). In addition, D2 -/- mice show a variety of other ethanol phenotypes, including decreased ethanol-induced ataxia using a grid assay (Phillips et al., 1998; Palmer et al., 2003), decreased ethanol-induced conditioned place preference (Cunningham et al., 2000), and decreased operant ethanol self-administration in a lever pressing assay (Risinger et al., 2000). While one study found no effect on locomotor activity in D2 -/- mice by ethanol (Phillips et al., 1998), other studies have found D2 -/- mice to show an enhancement of ethanol-stimulated activity relative to control mice (Palmer et al., 2003; Cunningham et al., 2000); this apparent discrepancy was explained as being due to differences in the genetic backgrounds of the mice used in these various studies.

Dopamine D3 and D4 receptor subtype knockout mice have also been generated and studied. While D3 deficient mice show increased sleep time in the LORR assay and increased severity of handling-induced convulsions in an ethanol-withdrawal assay (Narita et al., 2002), they show normal levels of ethanol consumption in a two-bottle choice assay, normal levels of ethanol-induced conditioned place preference, and normal levels of operant ethanol self-administration in a lever pressing assay (Boyce-Rustay and Risinger, 2003). D4 receptor deficient mice (D4 -/-) also show normal levels of ethanol consumption and preference in a two-bottle choice assay (Falzone et al., 2002), but increased ethanol-induced stimulation of locomotor activity in an open field assay (Rubinstein et al., 1997) and slightly increased sensitivity to the anxiolytic effects of ethanol, as measured in an elevated plus maze (Falzone et al., 2002). Thus, it appears that

dopamine may mediate its various effects on ethanol-induced behaviors through distinct receptor subtypes.

Studies of knockout mice deficient in the dopamine transporter (DAT -/-), the primary transporter through which dopamine is removed from the synaptic cleft, have yielded somewhat conflicting results. In one study, it was found that DAT -/- mice show decreased latency to lose their righting reflex in the LORR assay and decreased alcohol consumption and preference in females only in the two-bottle choice assay (Savelieva et al., 2002), while a second study found that deletion of the DAT gene results in increased ethanol consumption (but not preference) specifically in males (Hall et al., 2003). (Evaluation of ethanol-induced LORR was not performed in this second study.) The reason for this discrepancy is not clear, although, again, the authors suggest that differences in genetic background may have contributed to the differences in observed phenotypes. Mice deficient in the vesicular monoamine transporter 2 (VMAT2), which is involved in the vesicular storage of dopamine and serotonin into synaptic vesicles, were also studied and male VMAT2 -/- mice were found to have increased ethanol consumption, but not increased ethanol preference, in a two-bottle choice assay (Hall et al., 2003).

Lastly, mice deficient for DARPP-32 (dopamine and adenosine 3',5'-monophosphate-regulated phosphoprotein, 32 kDa), a downstream protein of the dopamine receptor that is important for the regulation of dopaminergic signaling (Greengard et al., 1999) were also tested for ethanol behaviors. While DARPP-32 -/- mice were found to have increased ethanol-induced locomotor activation, they show decreased ethanol-induced conditioned place preference, decreased ethanol self-

administration in a lever pressing assay, and normal ethanol-induced conditioned taste aversion (Risinger et al., 2001), suggesting that this downstream component of dopaminergic signaling may play a role in mediating the rewarding effects of ethanol.

E.) Serotonin / Serotonin Receptors

Studies *in vitro* have found that ethanol potentiates ion currents through the 5-hydroxytryptamine$_3$ (5-HT$_3$)-type serotonin receptor (Lovinger and White, 1991) and increases the probability of 5-HT$_3$ ion channel opening (Lovinger et al., 2000). Mice overexpressing the 5-HT$_3$ receptor (5-HT$_3$R) in the forebrain have been generated and tested in a variety of ethanol behavioral assays. 5-HT$_3$R overexpressing transgenic mice have decreased ethanol preference in a two-bottle choice assay (Engel et al., 1998; Metz et al., 2006), and increased ethanol-induced locomotor activity compared to controls in the open field assay (Engel and Allan, 1999; Metz et al., 2006), but do not differ from controls in terms of ethanol-induced sleep time in the LORR assay (Engel and Allen, 1999) or ethanol-induced aggression in male mice (McKenzie-Quirk et al., 2005). Interestingly, while the increased ethanol-induced locomotor activation of 5-HT$_3$R overexpressing mice is consistent across several genetic backgrounds, the ethanol consumption phenotype in the two-bottle choice assay is background specific, underscoring the importance of considering genetic background when testing ethanol-induced behavioral phenotypes (Metz et al., 2006).

Serotonin mediates its effects by acting on many different serotonin receptor subtypes in the CNS (Barnes and Sharp, 1999), and studies of transgenic mice bearing manipulations in various other aspects of serotonin signaling have been generated and

studied. For example, mice bearing a null mutation of the serotonin 6 receptor (5-HT_6R -/-) have been studied for their ethanol-induced phenotypes. 5-HT_6R -/- mice were found to show reduced sensitivity to ethanol-induced ataxia on the rotarod and reduced sensitivity to the sedative/hypnotic effects of ethanol as measured in the LORR assay, but these mice do not differ from controls when tested for ethanol consumption in the two-bottle choice assay or for ethanol-induced hypothermia (Bonasera et al., 2006).

Mice lacking the 5-HT_{1B} receptor gene (5-HT_{1B}R -/-) have also been tested in various ethanol assays and found to show increased ethanol consumption in the two-bottle choice assay, decreased ethanol-induced ataxia and slower ethanol tolerance development as measured in a grid assay, and normal levels of handling-induced convulsions in an ethanol withdrawal assay (Crabbe et al., 1996). 5-HT_{1B}R -/- mice show virtually no ethanol-induced conditioned place preference, but develop normal levels of ethanol-induced conditioned taste aversion, leading to the hypothesis that 5-HT_{1B} receptors may be important for mediating ethanol's rewarding, but not aversive, effects (Risinger et al., 1996). It is noteworthy that in a recent paper by Bouwknecht et al., the authors failed to replicate the two-bottle choice assay results of Crabbe et al., 1996; the reasons for this discrepancy are not clear (Bouwknecht et al., 2000). In addition, it was found that testing of the same strain of 5-HT_{1B}R -/- mice for ethanol-induced behaviors in different geographical laboratories (Albany, New York; Edmonton, Alberta, Canada; and Portland, Oregon) yielded different phenotypes, even when environmental variables and test protocols and apparati were rigorously controlled, suggesting that the results observed with these 5-HT_{1B}R -/- mice should be interpreted with a certain measure of caution (Crabbe et al., 1999).

In recent years, knockout mice lacking the serotonin transporter (5-HTT -/-) have also been developed and tested for ethanol phenotypes. 5-HTT -/- mice have decreased ethanol intake in a two-bottle choice assay (Kelai et al., 2003; Boyce-Rustay et al., 2006), increased sensitivity to the sedative/hypnotic effects of ethanol in a LORR assay (Daws et al., 2006; Boyce-Rustay et al., 2006) and increased ethanol-induced locomotor incoordination on the rotarod (Boyce-Rustay et al., 2006), but show normal levels of ethanol-induced hypothermia (Boyce-Rustay et al, 2006), ethanol-induced conditioned place preference (Boyce-Rustay et al., 2006), and ethanol-induced "pro-depressive-like" behaviors in a tail suspension test (Boyce-Rustay et al., 2006). Similarly, pharmacological blockade of 5-HTT using the 5-HT re-uptake inhibitor fluoxetine also reduces ethanol intake in the two-bottle choice assay (Kelai et al., 2003) and increases ethanol-induced sleep time in the LORR assay (Daws et al., 2006). Interestingly, Daws et al. also found that physiologically relevant doses of ethanol inhibit clearance of serotonin from extracellular fluid in the mouse hippocampus, and that this effect appears to occur via an uncharacterized mechanism independent of the serotonin transporter (Daws et al., 2006).

F.) Opiod peptides / Opiod Receptors

It has been known for quite some time that various opiod receptor antagonists can reduce ethanol consumption in humans and other animals (Myers et al., 1986; Volpicelli et al., 1992; Rodefer et al., 1999). Indeed, the nonselective opiod receptor antagonist naltrexone is one of the few effective pharmacologic treatments existing for alcoholism in humans (Volpicelli, 2001). Thus, in recent years there have been a number of studies of

ethanol-related behaviors in knockout mice deficient for the various major classes of opiod receptors (μ,δ, and κ) and their endogenous ligands.

Ethanol behavioral assays performed on μ-opiod receptor knockout (MOR -/-) mice have shown that these mice consume less ethanol than wild-type mice in a two-bottle choice assay (Roberts et al., 2000; Hall et al., 2001; Becker et al., 2002; Ghozland et al., 2005), exhibit decreased ethanol-induced locomotor activity (Hall et al., 2001; Ghozland et al., 2005), develop decreased ethanol-induced conditioned place preference (Hall et al., 2001), and develop decreased operant ethanol-self administration in nosepoke and lever press operant assays (Roberts et al., 2000). Studies regarding the anxiolytic-like effects of ethanol in μ-opiod receptor knockout mice have yielded conflicting results: one study found that ethanol-induced anxiolytic-like effects were blocked in MOR -/- mice when measured in the light-dark transfer test (Ghozland et al., 2005), while another study using the elevated plus-maze did not find a difference between MOR -/- mice and wild-type mice (LaBuda and Fuchs, 2001).

One study has been published studying the ethanol-related behaviors in δ-opiod receptor knockout mice. Unlike in MOR -/- mice, it was found that δ-opiod receptor knockout mice show increased preference for ethanol in a two-bottle choice assay and self-administer more ethanol than wild-type mice in a lever-pressing operant self-administration paradigm (Roberts et al., 2001), suggesting that the δ-opiod receptor may normally function to inhibit the reinforcing effects of ethanol.

Recently, ethanol related studies have also been performed on κ-opiod receptor knockout (KOR -/-) mice. While KOR -/- mice showed normal ethanol-induced locomotor activity when tested in an open-field locomotor assay, they showed decreased

ethanol consumption in a two-bottle choice assay (Kovacs et al., 2005). Interestingly, KOR -/- mice were also shown to have enhanced ethanol-evoked dopamine levels in the nucleus accumbens (Zapata and Shippenberg, 2006), although how this relates to the ethanol behaviors observed in KOR -/- mice is not clear.

Several studies have also been published on mice deficient for the endogenous opiods β-endorphin and enkephalin. Mice homozygous for a targeted deletion in the preproenkephalin gene, which codes for a precursor peptide from which the μ- and δ-opiod receptor agonist enkephalins (met^5- and leu^5-enkephalin) are derived, show no difference from wild-type mice in ethanol consumption or preference when measured in a two-bottle choice assay or a conditioned place preference assay, suggesting that endogenous enkephalins may not be involved in mediating ethanol consumption patterns or the rewarding effects of ethanol (Koenig et al., 2002). Somewhat surprisingly, two studies of mice deficient in β-endorphin (BE) both showed that BE knockout mice display increased, rather than decreased, ethanol intake as measured in the two-bottle choice assay (Grisel et al., 1999; Grahame et al., 2000). The authors suggest that this increased ethanol drinking behavior may be attributable to some unknown homeostatic compensatory reaction to the global lack of β-endorphin expression (Grisel et al., 1999).

In summary, it appears that the behavioral effects of ethanol are mediated through multiple opiod receptor subtypes, with the μ and κ subtypes normally functioning to promote the reinforcing effects of ethanol, and with the δ subtype normally functioning to inhibit ethanol's reinforcing effects.

G.) Cannabinoids / Cannabinoid CB1 Receptor

One of the newest targets of ethanol research is the cannabinoid (CB1) receptor. Known to most neuropsychopharmacologists as the primary central nervous system receptor for Δ9-tetrahydrocannabinol (THC), the major psychoactive component of marijuana (reviewed in Wilson and Nicoll, 2002), the CB1 receptor has, within the past 5 years, become the target of intense study by ethanol researchers. Several studies of CB1 knockout mice (CB1 -/-) have shown that CB1 deficient mice display reduced voluntary ethanol consumption in the two-bottle choice assay (Hungund et al., 2003; Poncelet et al., 2003; Naassila et al., 2004; Lallemand and de Witte, 2005; Thanos et al., 2005), increased sensitivity to the sedative/hypnotic effects of ethanol as measured in a loss of righting reflex (LORR) assay (Naassila et al., 2004), decreased ethanol-induced conditioned place preference (Houchi et al., 2005; Thanos et al., 2005), and increased ethanol withdrawal severity as measured by seizure behaviors during withdrawal (Naassila et al., 2004). CB1 deficient mice were also found to completely lack alcohol-induced dopamine release in the nucleus accumbens (Hungund et al., 2003) and were found to display an overexpression of striatal dopamine D2 receptors (Houchi et al., 2005). Similarly, wild-type mice infused with the CB1 receptor antagonist SR141716 (Rinaldi-Carmona et al., 1994) also show significantly reduced ethanol intake in the two-bottle choice assay; this effect can be also elicited by local infusion of the CB1 receptor antagonist into the nucleus accumbens (Caille et al., 2007). Conversely, FAAH knockout mice, which lack the enzyme fatty acid amidohydrolase (FAAH) and are consequently severely impaired in their ability to degrade the endogeous cannabinoid anandamide (Devane et al., 1992; Cravatt et al., 1996), show increased ethanol intake and preference as measured in the two-choice bottle assay and are less sensitive to the sedative/hypnotic

effects of acute ethanol as measured in the LORR assay (Basavarajappa et al., 2006). Interestingly, while administration of the cannabinoid receptor agonist HU-210 (Martin-Calderon et al., 1998) does not affect ethanol self-administration in a lever-pressing assay, administration of the anandamide transport inhibitor AM404 (Beltramo et al., 1997) significantly reduces ethanol self-administration in this assay (Cippitelli et al., 2007).

It is worthy to note that contrary to the results from the studies I've described above in CB1 -/- mice, Racz et al. found that CB1 -/- mice are normal for acute ethanol-induced behaviors, as measured in an open-field tests and an elevated zero maze, as well as for ethanol preference, as measured in the two-bottle choice assay (Racz et al., 2003). The same group also found that their CB1 -/- mice show no ethanol withdrawal (seizure) symptoms (Racz et al., 2003). The reasons for the apparent discrepancies between this study and the ones described above are unknown, although it has been suggested that they may be due to differences in the ethanol administration procedures and / or the genetic backgrounds of the mice tested (Naassila et al., 2004).

H.) Neuropeptide Y (NPY)

Neuropeptide Y (NPY), a 36 amino acid neuromodulator that is broadly expressed throughout the nervous system (Dumont et al., 1992), has been well studied for its roles in regulation of feeding behavior (Levine et al., 1984) and anxiety-associated behaviors (Heilig et al., 1992). NPY has been shown to alter ethanol behaviors in mice, as it was found that NPY deficient (NPY -/-) mice show increased ethanol consumption in a 2-bottle choice assay (Thiele et al., 1998), increased sensitivity to ethanol-induced

locomotor activation (Thiele et al., 2000a), as well as reduced recovery time from ethanol-induced sleep in a loss of righting reflex (LORR) assay (Thiele et al., 1998). Conversely, transgenic mice overexpressing NPY show decreased ethanol preference and increased sleep time in the LORR assay (Thiele et al., 1998). These altered ethanol induced behaviors were observed in NPY -/- mice on a mixed C57BL/6J x 129/SvEV background, but were not apparent when tested in NPY -/- mice on an inbred 129/SvEv background, emphasizing that expression of these phenotypes is sensitive to genetic background (Thiele et al., 2000a). A recent study also suggests a role for NPY in protecting against anxiety-like behaviors induced by ethanol withdrawal, as NPY -/- mice show elevated anxiety-like behavior, as measured in an elevated plus maze, during withdrawal after a six-day exposure to ethanol in their diets (Sparta et al., 2007).

In mice, NPY is known to act through multiple receptor subtypes (Y1, Y2, Y4, Y5, and Y6), which couple to heterotrimeric G-proteins that inhibit cAMP production (Palmiter et al., 1998). Several studies have aimed to determine which of these receptors are responsible for transducing the NPY signal in order to modulate ethanol-induced behaviors. While NPY Y1 receptor knockout mice (NPY Y1 -/-) show normal ethanol-induced ataxia on the rotarod test, they show increased ethanol consumption in a 2-bottle choice assay, as well as reduced sensitivity to the sedative effects of ethanol in a LORR assay, suggesting that NPY signaling through the Y1 receptor regulates some, but probably not all, of the ethanol behaviors regulated by NPY (Thiele et al., 2002). NPY Y2 receptor knockout mice display less promising phenotypes: they show normal sensitivity to ethanol-induced sedation and decreased ethanol consumption in a 2-bottle choice assay when tested on a mixed 129/SvJxBalb/cJ background, but show normal

levels of ethanol consumption when tested on a Balb/cJ background (Thiele et al., 2004). Results with NPY Y5 (Y5 -/-) knockout mice are similarly perplexing: Y5 -/- mice show normal ethanol-induced locomotor activity and normal voluntary ethanol consumption, but display increased sleep time in the LORR assay (Thiele et al., 2000a). The authors suggest that perhaps knockout of certain NPY receptors such as the Y2 and Y5 receptors may induce a compensatory up-regulation of other NPY receptors, such as the Y1 receptors, thus accounting for some of the somewhat confusing phenotypes observed in Y2 -/- and Y5 -/- mice, although this hypothesis has not been rigorously tested.

Interestingly, the NPY signaling pathway has been implicated in ethanol-induced behaviors in *C. elegans* and *Drosophila* as well. A study in roundworms with varying functional levels of NPR-1, an NPY receptor-like protein, show that NPR-1 negatively regulates the development of acute ethanol tolerance in *C. elegans* (Davies et al., 2004). In flies, acute ethanol sensitivity can be modified by increasing or decreasing expression levels of the fly homolog of NPY, NPF, or of its receptor, NPFR1 (Wen et al., 2005 – See Part 2 of this introduction). It is interesting to note that other modulators of appetitive behaviors, including leptin (Blednov et al., 2004b) and insulin (Corl et al., 2005) have also been implicated in regulating ethanol-induced behaviors.

I.) Adenosine / Adenosine Receptors

Extracellular adenosine functions as an important neuromodulatory signaling molecule in the CNS, acting through four G-protein-coupled receptor subtypes: the A_1, A_{2A}, A_{2B}, and A_3 receptors (Fredholm et al., 2001). *In vitro* studies dating back more than twenty years provided the first link between the adenosine receptor and ethanol signaling,

when it was shown that adenosine receptor-stimulated cAMP levels increase significantly when cultured neuroblastoma-glioma hybrid cells are acutely exposed to ethanol (Gordon et al., 1986). Treatment with the adenosine receptor antagonist BW A1434U completely blocks these intracellular increases in cAMP levels (Sapru et al., 1994). Further cell culture studies suggested a mechanism for how ethanol may potentiate adenosine signaling, when it was shown that acute ethanol exposure selectively inhibits the type 1 equilibrative nucleoside transporter (ENT1), resulting in increased levels of extracellular adenosine (Nagy et al., 1990).

An adenosine A_{2A} receptor knockout mouse (A_{2A} R -/-) has been generated and found to have several ethanol behavioral phenotypes. A_{2A} R deficient mice show increased ethanol preference in a two-bottle choice assay (Naassila et al., 2002), decreased sensitivity to the sedative/hypnotic effects of ethanol in a LORR assay (Naassila et al., 2002; El Yacoubi et al., 2003), and decreased sensitivity to ethanol-induced hypothermia (Naassila et al., 2002), while displaying equivalent levels of tolerance development to ethanol-induced hypothermia compared to controls (Naassila et al., 2002), and reduced severity of handling-induced convulsions during ethanol withdrawal (El Yacoubi et al., 2001). Pharmacological treatments of wild-type mice with A_{2A} R antagonists or agonists have yielded results that are harmonious with those found in A_{2A} R -/- mice. Treatment with the selective A_{2A} R antagonist ZM 241385 causes mice to be less sensitive to ethanol-induced hypothermia (Naassila et al., 2002) and to show reduced severity of handling-induced convulsions during ethanol withdrawal (El Yacoubi et al., 2001). Another selective A_{2A} R antagonist, SCH 58261, shortens the duration of sleep time in the LORR assay, as does the non-selective adenosine receptor antagonist

caffeine, while treatment with dipyridamole, an inhibitor of adenosine uptake, causes an increase in the duration of sleep time in the LORR assay (El Yacoubi et al., 2003).

As *in vitro* studies had shown that acute ethanol exposure selectively inhibits the nucleoside transporter ENT1, resulting in increased levels of extracellular adenosine (Nagy et al., 1990), ENT1 knockout mice (ENT1 -/-) were created and studied for their ethanol phenotypes. Similar to the results seen for A_{2A} R -/- mice, ENT1 -/- mice also show increased ethanol preference in the two-bottle choice assay, decreased sleep time in the LORR assay, and decreased ethanol-induced ataxia as measured on the rotarod (Choi et al., 2004). Interestingly, in the same study it was found that treatment of ENT1 -/- mice with the A_1 receptor (A_1R) agonist, N^6-cyclopentyladenosine (N^6-CPA), is sufficient to block the increased ethanol consumption phenotype of ENT1 -/- mice in the two-bottle choice assay (Choi et al., 2004). Ethanol studies of A_1 receptor knockout mice have not yet been reported. However, pharmacological manipulations of A_1R activity using A_1R agonists or antagonists suggest that the A_1R may also play a role in mediating ethanol's behavioral effects. For example, intra-striatal (Dar, 2001) or intra-cerebellar (Dar, 1997) micro-infusion of the selective A_1R agonist N^6-cyclohexyladenosine (CHA) results in an increase in ethanol-induced locomotor ataxia, while intra-striatal micro-infusion of the selective A_1R antagonist DPCPX results in the opposite phenotype, decreased ethanol-induced locomotor incoordination (Dar, 2001). It will be interesting to discover if further insights can be gleaned from studies of A_1R knockout mice, as well as from studies of mice deficient for other adenosine receptor subtypes.

J.) The cAMP / PKA Signaling Pathway

The cyclic adenosine monophosphate (cAMP) / cAMP-dependent protein kinase (PKA) pathway has been implicated in regulating a variety of life processes, including neural plasticity associated with learning and memory (Kandel and Schwartz, 1982; Wong et al., 1999), sensitization in nociception (Taiwo and Levine, 1991), and sensitivity to drugs of abuse (Moore et al., 1998; Andretic et al., 1999). The canonical cAMP / PKA signaling cascade has been well studied and described (Kandel et al., 2000). G-protein coupled receptor activation by ligand binding results in the activation of the stimulatory G protein, Gs. Activated Gs then stimulates the integral membrane protein adenylate cyclase (AC), which in turn catalyzes the conversion of ATP to cAMP. cAMP has various intracellular targets, but its major target of action is PKA, a holoenzyme composed of a regulatory (R) subunit homodimer and two catalytic (C) subunits (Brandon et al., 1997). Binding of cAMP to the regulatory subunits of PKA results in the release of the catalytic PKA subunits, freeing the latter to phosphorylate downstream targets.

Studies dating back over two decades have shown that ethanol stimulates adenylate cyclase activity and increases cAMP production *in vitro* (Rabin and Molinoff, 1983; Saito et al., 1985; Rabin et al., 1992), but it wasn't until relatively recently that the roles of the cAMP / PKA pathway in regulating ethanol-induced behaviors were explored *in vivo*. These behavioral studies were pioneered in *Drosophila*, where it was found that mutations in various components of the cAMP / PKA signaling cascade, including AC and the catalytic subunit of PKA, resulted in increased sensitivity to the intoxicating effects of ethanol (Moore et al., 1998; Refer to Part 2 of this introduction for more details). Since that study in *Drosophila*, multiple knockout and transgenic studies have

been conducted in mice, confirming a role for the cAMP / PKA pathway in regulating ethanol behaviors in mammals as well. For example, mice deficient in the adenylate cyclase isoforms AC1 (AC1 -/-) and AC8 (AC8 -/-) have been developed and studied (Maas et al., 2005). AC1 -/- mice show increased sensitivity to ethanol-induced sedation in the LORR assay, but display wild-type levels of ethanol consumption in the two-bottle choice assay and wild-type levels of ethanol-induced ataxia as measured in a variety of assays. In these same behavioral assays, AC8 -/- mice show only a very mild increase in sensitivity to ethanol-induced sedation, but display a significant decrease in ethanol consumption and wild-type levels of ethanol-induced ataxia. Double knockout mice with a genetic deletion of both AC1 and AC8 show both increased sensitivity to ethanol-induced sedation as well as decreased voluntary ethanol consumption. In other studies, where levels of the G protein subunit Gsα were manipulated, it was found that mice heterozygous for a deletion in the Gsα gene (Gsα +/-) show decreased ethanol consumption in the two-bottle choice assay (Wand et al., 2001), increased sensitivity to the sedative effects of ethanol in the LORR assay (Wand et al., 2001; Yang et al., 2003) and decreased tolerance to the sedative and hypothermic effects of ethanol (Yang et al., 2003). Conversely, transgenic mice overexpressing a constitutively active form of Gsα (Gsα Q227L) in the forebrain are less sensitive to the sedative/hypnotic effects of ethanol in the LORR assay, although they do not show a difference compared to wild-type mice when measured for ethanol consumption in the two-bottle choice assay (Wand et al., 2001).

In *Drosophila*, a loss of function mutant in the gene *amnesiac*, which codes for a peptide bearing homology to the pituitary adenylate cyclase-activating polypeptide

(PACAP) of mammals, showed altered sensitivity to ethanol intoxication (Moore et al., 1998). Knockout studies in mice have been carried out to study the effects of deleting PACAP or one of its receptors, PAC1. While mice deficient for PACAP (PACAP -/-) show ethanol phenotypes, manifested as decreased duration of sleep time in the LORR assay and decreased ethanol-induced hypothermia (Tanaka et al., 2004), knockout mice deficient for PAC1 (PAC1 -/-) do not show an altered sensitivity to ethanol-induced sedation in the LORR assay (Otto et al., 2001), suggesting that PACAP may mediate its effects on ethanol-induced sedation through a different receptor. It remains to be seen whether these PACAP -/- or PAC1 -/- mice will show ethanol phenotypes if tested in other ethanol behavioral assays.

Various pharmacological, knockout, and transgenic mouse studies have been performed to assess the contribution of the cAMP-dependent protein kinase (PKA) itself in regulating ethanol behaviors. While results from these studies clearly implicate a role for PKA in mediating the behavioral effects of ethanol, they don't always appear to "point in the same direction." Reduction of PKA activity in the forebrain through the expression of R(AB), a dominant inhibitory isoform of the regulatory subunit of PKA (Abel et al., 1997), causes mice to consume less ethanol in a two-bottle choice assay and become more sensitive to the sedative/hypnotic effects of ethanol in a LORR assay (Wand et al., 2001). Conversely, intracerebroventricular (ICV) injection of the specific PKA inhibitor KT 5720 dose-dependently decreases sensitivity to ethanol-induced sedation in the LORR assay, decreases ethanol-induced locomotor impairment on the rotarod, and reduces the inhibitory effects of ethanol on NMDA-induced convulsions (Lai et al., 2007).

Mice lacking a major PKA regulatory subunit, RIIβ, have been generated (RIIβ -/-) and studied for ethanol-induced behaviors. It was found that PKA RIIβ -/- mice show increased ethanol consumption in the two-bottle choice assay (Thiele et al., 2000b; Fee et al., 2004; Ferraro et al., 2006), decreased sensitivity to the sedative effects of ethanol in the LORR assay (Thiele et al., 2000b; Fee et al., 2004), increased ethanol-induced acute locomotor activation and increased sensitivity to ethanol-induced locomotor sensitization in the open field arena (Fee et al., 2006), and decreased levels of operant self-administration of ethanol in females but not in males (Ferraro et al., 2006). Knockout mice lacking the PKA RIβ regulatory subunit and the PKA Cβ1 catalytic subunits were also tested for voluntary ethanol consumption but were found to have normal levels of ethanol consumption in the two-bottle choice assay compared to controls (Thiele et al., 2000b), suggesting that some, but not all, subunits of PKA are involved in mediating ethanol-induced behaviors in mammals.

K.) Protein Kinase C (PKC)

The protein kinase C (PKC) family of kinases, activated by calcium, diacylglycerol, and a variety of other lipids, is encoded by nine genes divided into three subfamilies: conventional (α, β, γ), novel (δ,ε,η,θ), and atypical (ζ and ι/λ) (Newton and Ron, 2007). Of these PKC family members, the ethanol phenotypes of PKCγ and PKCε null mice have been characterized and will be reviewed in this section.

Null mutant mice lacking the γ isoform of PKC (PKCγ -/-) have been generated and extensively studied for ethanol-induced behaviors. PKCγ -/- mice have reduced sensitivity to the hypnotic/sedative effects of ethanol in a LORR assay (Harris et al.,

1995; Proctor et al., 2003), decreased ethanol-induced hypothermia (Harris et al., 1995), increased ethanol intake in a two-bottle choice assay (Bowers and Wehner, 2001), attenuated ethanol-induced anxiolytic behaviors in the elevated plus-maze (Bowers et al., 2001), and male PKCγ -/- mice exhibit increased sensitivity to the locomotor activating effects of ethanol in an open-field arena (Hix et al., 2003). In addition, PKCγ -/- mice also fail to develop chronic ethanol tolerance after ten days on an ethanol liquid diet, although it should be noted that this tolerance phenotype is genetic background sensitive (Bowers et al., 1999). Interestingly, ethanol-induced enhancement of $GABA_A$ receptor mediated inhibitory postsynaptic currents (IPSCs) was found to be attenuated in PKCγ deficient mice (Proctor et al., 2003; Harris et al., 1995), suggesting a role for PKCγ in modulating ethanol-induced enhancement of $GABA_A$ receptor function.

Conversely, studies of null mutant mice lacking the ε isoform of PKC (PKCε -/-) have shown that these mice, in several assays, exhibit ethanol behaviors that are opposite to those seen in PKCγ -/- mice. PKCε -/- mice show increased sensitivity to the hypnotic/sedative effects of ethanol in a LORR assay (Hodge et al., 1999; Choi et al., 2002; Proctor et al., 2003), decreased ethanol intake in a two-bottle choice assay (Hodge et al., 1999; Choi et al., 2002), reduced operant ethanol self-administration in an operant lever pressing paradigm (Olive et al., 2000), and increased ethanol-induced locomotor activity (Hodge et al., 1999). While development of chronic ethanol tolerance appears to be intact in PKCε -/- animals, these mice show reduced acute functional ethanol tolerance as measured using the stationary dowel test (Wallace et al., 2007). Using an inducible gene strategy, Choi et al. found that adult specific expression of PKCε in the basal forebrain, amygdala, and cerebellum of PKCε deficient mice is sufficient to rescue the

ethanol preference and LORR phenotypes seen in PKCε -/- mice, providing insight into the temporal and spatial requirements for PKCε in the regulation of ethanol sensitivity (Choi et al., 2002). Conversely to what was found in PKCγ -/- mice, ethanol-induced enhancement of $GABA_A$ receptor mediated IPSCs is markedly potentiated in PKCε -/- mice (Proctor et al., 2003; Hodge et al., 1999). Roles for PKCε in other pathways have been described as well: one study showed that PKCε is necessary for modulation of ethanol consumption by mGluR5 (the type 5 metabotropic glutamate receptor) (Olive et al., 2005) and another showed that ethanol-induced increases in extracellular dopamine levels are absent in the nucleus accumbens of PKCε deficient mice (Olive et al., 2000).

L.) Growth Factors

Several studies have implicated a role for growth factors in mediating ethanol –induced behaviors in mice. Overexpression of transforming growth factor-α (TGF-α), for example, was found to increase ethanol consumption in TGF-α transgenic mice as measured in a two-bottle choice assay (Hilakivi-Clarke and Goldberg, 1995).

A role for insulin-like growth factor I (IGF-I) has also been described (Pucilowski et al., 1996). In this study, it was found that transgenic mice overexpressing IGF-I display decreased sensitivity to the sedative/hypnotic effects of ethanol as measured in a LORR assay and fail to acquire chronic ethanol tolerance after repeated i.p. injections of ethanol as measured in the LORR assay. Conversely, transgenic mice overexpressing IGF binding protein 1, which inhibits the *in vivo* actions of IGF-I (D'Ercole et al., 1994), show the opposite phenotypes: increased sleep time in the LORR assay and increased chronic ethanol tolerance as measured in the LORR assay. Ethanol-induced ataxia was

also measured using a rotarod assay, but no significant differences were found between the transgenic animals and controls.

In addition, brain-derived neurotrophic factor (BDNF), an important mediator for neuronal survival, has also been shown to regulate ethanol behaviors. BDNF heterozygous mice (BDNF +/-) were tested for a variety of ethanol behaviors and were found to develop increased conditioned place preference for ethanol, increased acute ethanol-induced locomotor activity, increased sensitization to ethanol-induced locomotor activity, and increased ethanol intake after a 2-week period of ethanol deprivation (McGough et al., 2004). Interestingly, ethanol administration increased BDNF expression both *in vitro* and *in vivo*, with BDNF expression levels specifically increased in the dorsal striatum. In studies to define a signaling pathway through which BDNF might mediate ethanol-induced behaviors, the authors found that the scaffolding protein RACK1 positively regulates the expression levels of BDNF, and that increasing brain levels of RACK1 through i.p. injection of a Tat-RACK1 fusion protein results in reduced ethanol consumption and sensitization (McGough et al., 2004). In a subsequent study, Jeanblanc et al. found that ethanol- or RACK1 induced- increases in BDNF expression lead to increased expression levels of the dopamine D_3 receptor, a downstream target of BDNF, in the dorsal striatum (Jeanblanc et al., 2006). In the same study, the authors found that pharmacological inhibition of the BDNF receptor TrkB using K252a (Tapley et al., 1992) results in increased ethanol consumption in the two-bottle choice assay (Jeanblanc et al., 2006). From their collective data, the authors propose a courageous model in which ethanol exposure- or RACK1-induced increases in BDNF expression result in increased expression of the dopamine D_3 receptor via a BDNF signaling cascade involving the

TrkB receptor. The resulting increased dopamine D_3 receptor activity is then hypothesized to negatively regulate ethanol intake.

Summary

It is interesting to note that until fairly recently, it was widely believed that ethanol's actions were effected primarily via its non-specific interactions with lipids in the cell membrane, through alterations of membrane fluidity (described in Harris, 1999). This "membrane fluidity" hypothesis has since fallen out of favor, as a wealth of pharmacological and transgenic studies, performed both *in vitro* and *in vivo*, have shown that ethanol's actions are instead mediated through the activation and inhibition of a variety of neurotransmitter receptors and ion channels, with downstream effectors including such intracellular signaling cascades as the cAMP / PKA signaling pathway. The generation and use of knockout and transgenic mice has greatly advanced the field of ethanol research, allowing scientists to address the individual contributions of specific neurotransmitter receptors and signaling pathways to various aspects of ethanol-induced behaviors. Many results of these studies, including those implicating the cannabinoid CB1 receptor in mediating ethanol-induced behaviors, have been uncovered within just the past 5 years, suggesting that the field of ethanol research is still far from being fully understood. While the studies performed using rodent models that I've described in this section have certainly provided invaluable insight into ethanol's many actions, recent studies using invertebrate model organisms, especially *Drosophila melanogaster*, have also proven to be extremely fruitful. I will describe the results from such ethanol studies using the fruit fly as a model system in the following section of this introduction.

Part II: Using *Drosophila* as a Model System for Drug Studies

A.) Introduction to *Drosophila* as a Model Organism

Reared and studied in the laboratories of geneticists for the past hundred years, the fruit fly *Drosophila melanogaster* has flown its way into the hearts of many a scientist and has established itself as one of the key model organisms used to understand the myriad processes underlying this mystery we call life. The advantages to using *Drosophila* as a model system are manifold (reviewed in St. Johnston, 2002). They are small, easy and inexpensive to raise, and are capable of generating hundreds upon hundreds of progeny within a short 10-day generation time. Equipped with a well-annotated genome bearing many genes with mammalian homologues/orthologues, the fruit fly lends itself well to mutagenesis for the purpose of forward genetic screening. A plethora of mutants can be generated in a relatively short period of time using various methods, including the feeding of ethyl methane sulfonate (EMS) or mobilization and random insertion of small transposable elements called P elements (Engels, 1983). The use of P element-mediated mutagenesis has become increasingly popular, as the exact insertion site of an inserted P-element can be easily tracked and identified through the use of such techniques as plasmid rescue and inverse polymerase chain reaction (inverse PCR). The fruit fly is also amenable to germ line transformation (Rubin and Spradling, 1982; Spradling and Rubin, 1982) and homologous recombination (Rong and Golic, 2000), and the development of visible chromosomal markers and balancer chromosomes facilitates the ease in which mutants can be created and characterized (Greenspan, 1997).

One of the most useful tools developed for use in *Drosophila* is the GAL4 / UAS binary system (Brand and Perrimon, 1993; Brand et al., 1994), which allows for the restricted spatial expression of a transgene of interest using any of the large number of GAL4 driver lines available (http://www.fly-trap.org/). Using this system, one can study the consequences of expressing a particular transgene of interest in a restricted subset of cells or cell types. In addition, through the GAL4 / UAS system, one can genetically ablate a subset of cells through the expression of cell death-promoting factors, such as *reaper* (McNabb et al., 1997), or inactivate subpopulations of neurons through the expression of tetanus toxin light chain (Sweeney et al., 1995) or a temperature-sensitive dynamin homologue, *shibire*^{ts} (Kitamoto, 2001). Endogenous gene expression can also be perturbed through the use of double-stranded RNA-mediated gene expression interference (RNAi) (Carthew, 2001; Kalidas and Smith, 2002).

In recent years, modifications to the GAL4 / UAS system that allow for temporal control over transgene expression have been developed. In one such system, commonly known as the P[Switch] system, transgene expression can be conditionally activated by feeding flies the progesterone receptor ligand RU486 (Osterwalder et al., 2001; Roman et al., 2001). In another such system, the TARGET system, the presence of a transgene encoding a temperature-sensitive GAL4 inhibitor, GAL80^{ts}, allows for GAL4 expression to be dependent on the temperature at which the flies are housed: GAL4 expression is turned off at lower temperatures such as 18°C (when GAL80^{ts} is active) and is turned on at higher temperatures such as 29°C (when GAL80^{ts} is inactive) (McGuire et al., 2004).

Contrary to what some naysayers may think, the tiny fruit fly is capable of executing quite a variety of complex behaviors (Sokolowski, 2001). Through the use of

forward genetic screens in *Drosophila*, researchers have successfully identified many of the genetic and biochemical substrates underlying a variety of complex behaviors including courtship, aggression, learning and memory, circadian rhythm-regulated behaviors, and drug-induced behaviors, among many others. Importantly, many of the substrates and pathways implicated in *Drosophila* have since been found to have conserved functions in mammals, validating the usefulness of *Drosophila* as a model organism for studying such complex behavioral processes. In the following sections I will describe in detail how *Drosophila* has been successfully used to study the mechanisms underlying drug-induced behaviors, with an emphasis on ethanol-induced behaviors and cocaine-induced behaviors.

B.) *Drosophila* as a Model System for Alcohol Research

Overview

The fruit fly, *Drosophila melanogaster*, is no stranger to alcohol (ethanol). Living their lives in close proximity to fermenting plant materials, where alcohol concentrations can reach relatively high levels of 3% or more (Guarnieri and Heberlein, 2002), fruit flies have undoubtedly evolved, over the millennia, genetic pathways to regulate their behavioral and physiological responses to this intoxicating drug. Until relatively recently, however, studies concerning ethanol and *Drosophila* had almost exclusively centered on the ability for fruit flies to withstand the toxic effects of ethanol (reviewed in Geer et al., 1993). These studies of "ethanol tolerance" (not to be confused with the functional ethanol tolerance to the intoxicating behavioral effects of ethanol

described later in this introduction) focused on studying the mechanisms of elimination of ethanol in *Drosophila*, highlighting the importance of such metabolic enzymes as alcohol dehydrogenase (ADH), and to a lesser extent, aldehyde dehydrogenase (ALDH) in the breakdown of ethanol in larvae and adults (Geer et al., 1993).

Within the past decade, however, *Drosophila melanogaster* has emerged as a model organism not simply for studying the processes that regulate the metabolism of ethanol, but also the genes and pathways that mediate sensitivity to the behavioral effects of ethanol exposure. Importantly, several of these genes and pathways have since been found to also regulate ethanol sensitivity in mammals as well, validating the use of this small but powerful model organism in studying the mechanisms underlying ethanol induced behaviors.

In this portion of the introduction, I will first describe the acute effects of ethanol on *Drosophila* behavior as well as the various paradigms and assays that have been developed to quantify these ethanol-induced behaviors. I will then discuss how these various assays have been used to identify specific genes and pathways involved in mediating ethanol sensitivity, as well as the various neuroanatomical loci that have also been shown to be important in regulating the ethanol response in *Drosophila*. I will also describe the use of *Drosophila* in studying functional ethanol tolerance, and what genes and pathways have been discovered to regulate this acquisition of behavioral resistance to multiple or continuous exposures of ethanol. Finally, I will discuss studies focusing on using *Drosophila* to study preference behaviors towards ethanol.

Acute Effects of Ethanol on *Drosophila* Behavior

i.) Description of Acute Ethanol Intoxication in *Drosophila*

The effects of ethanol on human behavior are all too familiar to many of us. Lower concentrations of ethanol induce disinhibition and euphoria, whereas higher concentrations of ethanol induce locomotor incoordiation, sluggishness, and eventual sedation, coma, and death. Within the past decade, the behavioral response of *Drosophila* to ethanol has been extensively studied and characterized (Moore et al., 1998; Singh and Heberlein, 2000; Wolf et al., 2002). Fascinatingly, *Drosophila* exhibit behavioral responses to alcohol that are similar to those of mammals at pharmacologically relevant concentrations of ethanol. As in mammals, low concentrations of ethanol induce locomotor activation, whereas higher ethanol concentrations induce locomotor incoordination, akinesis, and ultimately death (Wolf et al., 2002).

The locomotor behavioral response of *Drosophila* subjected to a continuous stream of ethanol vapor can be described in three phases (Wolf et al., 2002):

1.) Immediate and transient hyperactivity ("startle response")

2.) Sustained hyperactivity ("hyperactive phase")

3.) Locomotor incoordination and akinesis/sedation ("sedation phase")

The first phase of the ethanol-induced locomotor response, also known as the "startle" response, is characterized by a rapid and transient increase in locomotor speed that commences almost immediately after switching exposure from air to ethanol vapor and subsides in less than a minute. The magnitude of the startle response is dependent, up

to a point, on the concentration of ethanol vapor, with high ethanol concentrations inducing increases in both the peak velocity and the total movement during the startle phase (Cho et al., 2004). Antennectomized flies, lacking their third antennal segments, the major olfactory organs of *Drosophila* (Carlson, 1996), show a greatly attenuated startle response, indicating that the startle response is an olfactory mediated response induced by the novel odor of ethanol (Cho et al., 2004). Upon repeated exposures to pulses of ethanol vapors, the startle response gradually habituates, decreasing in magnitude over several pulses before reaching a plateau (Cho et al., 2004). Forward genetic screens for *Drosophila* mutants that exhibit altered levels of startle response habitutation (Wolf et al., 2007) as well as selective ablation of specific brain regions (Cho et al., 2004) have provided insight into the molecules and neuroanatomical substrates that underlie this relatively simple form of nonassociative learning.

Following the startle response is a transient quiescent phase followed by a sustained hyperactivity phase, also known as the hyperactive phase (Wolf et al., 2002). During the hyperactive phase, which lasts for several minutes (depending on the ethanol concentration being administered), the average walking speed of *Drosophila* again increases, often to as high a level as during the startle response. Unlike the startle response, the hyperactive phase is not attenuated by surgical removal of the antennae, indicating that this second phase of locomotor activation is likely not olfactory mediated (Wolf et al., 2002). In addition, unlike the startle response during which little to no ethanol has accumulated in the fly, the hyperactive response is associated with a measurable increase in internal concentrations of ethanol, with ethanol levels reaching approximately 20 mM at the peak of the hyperactive phase (Wolf et al., 2002). The

magnitude and duration of the hyperactive phase is inversely proportional to the ethanol concentration being delivered, with lower ethanol concentrations tending to result in a greater and longer lasting hyperactive response (Wolf et al., 2002).

The third and final phase of the ethanol induced locomotor response, the sedation phase, is associated with increased levels of internal ethanol concentration reaching approximately 40 mM (Wolf et al, 2002). During this phase, *Drosophila* exhibit a variety of behaviors commonly associated with heavy intoxication, including gradual to complete loss of postural control and eventual immobility, with flies often lying akinetic on their sides or backs (Wolf et al., 2002; A. Corl, personal observations). These sedated flies, while unable to walk or right themselves if upended, are not dead, as replacement of the ethanol vapors with a continuous humidified air stream results in the flies recovering over the course of several minutes from their ethanol induced akinesis (Wolf et al., 2002). Unsurprisingly, the onset of the sedation phase can be hastened by increasing the ethanol concentration being delivered to the flies: at the relatively low ethanol/air concentration of 50/100 (corresponding to a relative flow of 50 U of ethanol vapor and 100 U of humidified air) almost no flies are sedated at the end of a 20-minute exposure period (Wolf et al., 2002; A. Corl personal observations), whereas a higher ethanol/air concentration of 110/40 results in 50% of control flies experiencing sedation after approximately 15 minutes of ethanol vapor exposure (Rothenfluh et al., 2006).

ii.) Methods for Assaying the Acute Effects of Ethanol on *Drosophila* Behavior

I. Inebriometer

As an assay used to measure ethanol-induced loss of postural control in *Drosophila*, the inbriometer has proven itself as a useful tool for identifying genes, biochemical pathways, and neuroanatomical loci involved in mediating both acute behavioral responses to ethanol as well as those involved in tolerance development (Moore et al., 1998; Singh et al., 2000, Scholtz et al., 2000; Rodan et al., 2002; Berger et al., 2004; Scholz et al., 2005). Invented over two decades ago (Cohan and Hoffman, 1986; Weber, 1988), the inebriometer is an approximately four-foot long glass column filled with spaced, obliquely oriented mesh baffles. The temperature of the inebriometer column is kept constant at 20°C through the use of a water jacket encircling the column, and ethanol vapor is circulated through the interior of the column. Approximately 100 flies of a given genotype are introduced into the top of the inebriometer column. As the flies become intoxicated, they gradually lose postural control and fall down from baffle to baffle. Eventually, the intoxicated flies fall out, or "elute," from the bottom of the column, at which point they are counted in 3-minute time bins by an infrared beam monitor. From the elution profile data, which generally follow a normal distribution, one can calculate a mean elution time, or MET, for each genotype. The MET gives a measurement of a fly genotype's sensitivity to ethanol-induced loss of postural control: a high MET indicates lower sensitivity whereas a low MET indicates higher sensitivity.

II. Line Crossing Assay

An early, non-automated method used to quantify horizontal locomotion in *Drosophila*, the line crossing assay involves introducing flies into an airtight acrylic box covered with a grid of orthogonal lines spaced approximately 1.5 cm apart (Singh et al.,

2000; Bainton et al., 2000). After a rest period of 2 minutes in humidified air, the flies are given a continuous stream of ethanol vapor mixed with humidified air for a period of 20 minutes. Horizontal locomotion is determined by calculating the number of lines crossed per minute per fly, with the observer manually drawing out traces of each fly's movement on a map of the exposure chamber. To prevent flying in the chamber, flies' wings are clipped 24 hours before testing. This assay has proved useful for quantifying the changes in locomotor behavior associated with the hyperactive and sedation phases, but is certainly laborious and difficult to carry out using larger numbers of flies.

III. Turning Assay (Singh et al., 2000)

When observing fly locomotion in response to ethanol in the line crossing assay (see above), it was found that intoxicated flies not only move significantly faster than non-intoxicated flies during the hyperactive phase, but they also appeared to change directions more often (Singh et al., 2000). To quantify this ethanol-induced increase in turning behavior, a turning assay was developed (Singh et al, 2000). In this assay, individual flies are introduced into narrow perforated tubes (9 mm in length and 3 mm in diameter) with lines marking each 1 cm. The diameters of the tubes are narrow, only slightly wider than the size of a fly, forcing the enclosed flies to walk in either one of two directions. In the absence of ethanol vapors, flies tend to walk straight from one end of the tube to the other, with changes in directions confined mostly to the ends of the tubes. In the presence of ethanol vapors, however, turning behavior (measured as the number of turns per minute) increases dramatically, with maximal turning behavior reaching an approximately 12-fold increase over that of air exposed flies. Interestingly, the

hyperactive effects of ethanol on *Drosophila* locomotion are not observed when using the narrow, long turning assay tubes, although the decrease in locomotor behavior associated with the sedative phase is easily measured in this assay.

IV. Inebri-actometer

Another device developed to measure the ethanol-induced locomotor behavior of *Drosophila*, the inebri-actometer consists of 128 chambers fitted with emitter/detector photodiodes (Parr et al., 2001). Individual flies are introduced into each of the 128 chambers through which ethanol vapors are then circulated. Each time a fly blocks the infrared signal transmitted in each chamber, an attached computer records a movement for that particular fly. Using the inebri-actometer, Parr et al. were able to construct locomotor activity graphs for wild-type flies subjected to different ethanol vapor concentrations, observing both the hyperactive as well as sedative phases. While the inebri-actometer offers the ability to potentially screen through a large number of flies simultaneously, setting up each run can be a bit time consuming: the authors note that loading flies into the inebri-actometer can take 20-30 minutes, and it takes another 15 minutes for the flies to recover from carbon dioxide anesthesia (Parr et al., 2001).

V. Automated Motion-Tracking System / Booz-o-mat

In the automated motion-tracking system developed by Wolf *et al.*, 20 or more flies of the same genotype are introduced into a transparent exposure chamber and allowed to acclimatize in a stream of humidified air (Wolf et al., 2002). The air stream is subsequently replaced with a stream of continuous ethanol vapor, and the motion of the

flies is digitally recorded using a video camera mounted above the exposure chamber. The movies are saved onto a computer attached to the camera, and are later analyzed using the program DIAS (Dynamic Image Analysis System, Solltech, Oakdale, IA), which calculates the speed of the flies (expressed in mm/sec) at various time points during the ethanol exposure. Locomotor tracking profiles generated through the use of this tracking system clearly show the presence of the startle response, followed by the hyperactive phase and the sedation phase. Studies of single flies in the automated motion tracking system showed that flies move in bouts (short periods of activity punctuated with periods of inactivity) and that ethanol increases the lengths of these bouts of locomotor activity during the hyperactivity phase (Wolf et al., 2002).

For the purposes of screening large numbers of *Drosophila* mutants, Wolf *et al.* modified their tracking system to incorporate 8 smaller chambers instead of one large chamber. In this higher-throughput locomotor tracking system, called the "booz-o-mat," 25 flies of each genotype are introduced into perforated test tubes, which are then fitted into the 8-chambered booz-o-mat apparatus. Ethanol vapors are delivered simultaneously to the 8 perforated test tubes, and the movements of the flies in each tube are digitally recorded and analyzed as described above using DIAS.

VI. Sedation Assays

As flies accumulate high internal concentrations of ethanol, they lose postural control and become akinetic, or sedated (Wolf et al., 2002). Various groups have developed similar approaches to visually assaying these ethanol-induced sedation responses in *Drosophila*. In Park et al., 30 flies of each genotype are introduced into

perforated 50-ml Falcon tubes, through which humidified ethanol vapors are circulated (Park et al., 2000). Numbers of immobilized, sedated flies are counted in five-minute intervals by an observer and a sedation curve plotting the fraction of immobilized flies versus elapsed exposure time is generated. From these sedation profiles, a KO50, the time to immobilize 50% of flies, is calculated for the various genotypes and compared by statistics.

In Wen et al., 1 ml of the desired concentration of ethanol is applied to a folded Kimwipe tissue paper and laid on the bottom of a plastic fly bottle (Wen et al., 2005). Flies are added to the bottle, and the numbers of sedated flies that drop to the bottom to the bottle are counted at 5-minute intervals.

Urizar et al. designed a special apparatus used to test sensitivity to ethanol-induced sedation in *Drosophila* (Urizar et al., 2007). This apparatus consists of 8 clear plastic vials placed in a circular arrangement around a Plexiglass manifold through which pressurized ethanol vapors are circulated. Flies are introduced into the plastic vials and the percentage of sedated flies is recorded every 5 minutes. Urizar et al. score a fly as sedated if it has fallen onto its back or side and is motionless.

Rothenfluh et al. quantified sedation in *Drosophila* through a loss-of-righting (LOR) test (Rothenfluh et al., 2006). Flies are loaded into the 8-chambered booz-o-mat apparatus (see Section V. above) and subjected to a continuous stream of ethanol vapors. At 5-minute intervals the experimenter, blinded to the genotypes of the flies, lightly taps each tube and counts the flies unable to right themselves as being sedated. Time to 50% LOR is calculated through linear interpolation of the two time points around the median.

VII. Recovery Assays

Various groups have developed methods for quantitatively assaying recovery from ethanol-induced sedation. A simple but effective example of a recovery assay is described by Wen et al., in which 20 un-anesthetized females are placed in a plastic vial with a cotton plug (Wen et al., 2005). 1 ml of 100% ethanol is slowly added to the cotton plug, and over the course of the next 12 minutes, all flies become sedated (lay motionlessly) at the bottom of the vial. Subsequently, the ethanol soaked plug is replaced with a fresh, ethanol-free plug, and flies are allowed to recover from ethanol-induced sedation over the next 40 minutes. Numbers of recovered flies, as measured by recovery of climbing and flying behaviors, are recorded at 2-minute intervals.

The recovery assay described by Berger et al. involves introducing 8 groups of 25 male flies into each of eight 50-mL conical perforated Falcon tubes (Berger et al., 2004). After sedating the flies through exposure to a 60/40 EtOH/air stream of ethanol vapors, flies are transferred into empty fly vials and allowed to recover in air. Flies are scored as recovered if they are able to climb onto the side of the vial walls. Recovery is assayed each minute by a blinded observer, and a mean recovery time (MRT), the time at which 50% of the flies regained this negative geotaxis behavior, is calculated for each genotype.

Similarly, Cowmeadow et al. use a glass and Teflon device, the "inebriator," to administer ethanol vapors to twelve perforated plastic vials containing 10 fruit flies each (Cowmeadow et al., 2005; Cowmeadow et al., 2006). After all the flies in the vials are sedated (sedated flies being defined by the authors as "those flies lying on their backs or sides or those "facedown" with their legs splayed out in a nonstandard posture") the ethanol vapor stream is replaced with a humidified non-ethanol air stream and recovery is

assayed each minute thereafter. Flies are scored as recovered if they stand upright and appear to have regained postural control.

While most assays involving measurement of ethanol sensitivity in *Drosophila* rely on intoxicating the flies using ethanol vapors, Dzitoyeva et al. utilized a different method for delivery of the drug: injecting the ethanol (0.2 μl per fly) directly into CO_2-anesthetized flies using beveled glass pipettes (Dzitoyeva et al., 2003). Injected flies are loaded into the *Drosophila* Activity Monitoring System (Trikinetics, Waltham, MA) within 2 minutes of injection and movements are monitored using this system at 1-minute intervals. Recovery from anesthesia (aka "time to awakening") is defined by the authors as the first one-minute interval in which a fly produces 10 movements.

iii.) Genes and Biochemical Pathways Mediating Acute Ethanol Sensitivity in *Drosophila*

Over the past decade, various groups have utilized *Drosophila* as a model organism for studying the pathways that mediate acute ethanol sensitivity in the fruit fly. Most of these groups have used forward genetic approaches to screen for *Drosophila* mutants that show altered ethanol-induced behaviors, taking advantage of the fact that *Drosophila*, a proliferative, cheap, and easy to raise model organism in which mutations are easily generated and tracked, lends itself well to such forward genetic screens. A few groups have taken alternate approaches, however, using reverse genetic strategies such as RNA interference (RNAi) to target specific genes of interest (Dzitoyeva et al., 2003) as well as microarray experiments to gain insight into the alterations of gene expression that are induced by ethanol exposure (Urizar et al., 2007; Morozova et al., 2006). In this section I will discuss the various genes and biochemical pathways implicated utilizing

such forward and reverse genetic approaches as well as promising candidates isolated through microarray analysis.

I. EMS Screen: *barfly* and *tipsy*

One of the earliest forward screens for ethanol sensitivity mutants in *Drosophila* was performed by Singh and Heberlein using the inebriometer (Singh and Heberlein, 2000). Approximately 30,000 males, mutagenized through treatment with ethyl methane sulfonate (EMS), were screened in the inebriometer, and 19 strains with reduced ethanol sensitivity (defined as having a mean elution time (MET) greater than 125% of control) and 4 strains with increased ethanol sensitivity (defined as having an MET less than 78% of control) were isolated. Two of these mutants were selected for further characterization: mutant F48/*barfly* (*brf*) and mutant I79/*tipsy* (*tps*) were isolated due to their decreased (*brf*) and increased (*tps*) ethanol sensitivities, respectively. Neither of these mutants displays altered ethanol pharmacokinetics, indicating that the ethanol phenotypes observed are not simply due to alterations in the rate of ethanol absorption or metabolism. When tested in the line crossing assay, both *barfly* and *tipsy* flies hyperactivate similarly to controls. However, as ethanol exposure continues, *tipsy* flies display increased sensitivity to ethanol-induced sedation, whereas *barfly* mutants are resistant to ethanol-induced sedation. Interestingly, when tested in the turning assay, both mutants display increased turning behavior compared to controls. Although a major advantage of EMS mutagenesis is that it is relatively easy to generate a large number of mutants quickly, a major disadvantage is that it can be difficult to track and determine which genes are affected by the mutagenesis. As such, the molecular characterizations of the genes

disrupted in *barfly*, *tipsy*, and the other EMS mutants isolated in this EMS screen have remained elusive.

II. cAMP / PKA pathway: *cheapdate*

The initial study which put *Drosophila melanogaster* on the map as a model organism suitable for studying the genes and pathways regulating the behavioral responses to ethanol was published nearly a decade ago by Moore et al. (Moore et al., 1998). In this study, Moore et al. screened through a collection of ~5000 mutants carrying P element-induced X-linked mutations using the inebriometer and isolated an ethanol-sensitive mutant, *cheapdate* (*chpd*). The mutation in *cheapdate* was mapped to the gene *amnesiac* (*amn*), the protein product of which bears homology to the mammalian pituitary adenylyl cyclase activating peptide (PACAP) (Feany and Quinn, 1995), suggesting a role for the cyclic AMP/protein kinase A pathway in mediating the behavioral effects of ethanol in *Drosophila*.

Indeed, Moore et al. found that various mutations that are known to impair cAMP signaling, such as those in *rutabaga*, encoding the Ca^{2+}-calmodulin-sensitive adenylyl cyclase (Livingstone et al., 1984; Levin et al., 1992), and in DCO, encoding the major catalytic subunit of cAMP-dependent protein kinase (Lane and Kalderon, 1993), also increase ethanol sensitivity in the inebriometer, as does pharmacalogical treatment of control flies with the PKA inhibitor Rp-cAMPS (Rothermel and Botelho, 1988). A subsequent study by Park et al. demonstrated that the loss of function mutant *pka-RII* $^{EP(2)2162}$, which shows a 2-fold increased basal PKA activity and a 40% of normal cAMP-inducible PKA activity, is less sensitive to the sedating effects of ethanol as measured in

a sedation assay (Park et al., 2000). Taken together, these studies were among the first to show *in vivo* that the cAMP/PKA signaling pathway is involved in regulating the behavioral response to ethanol. Since then, various studies in mammals have confirmed the role of the cAMP/PKA pathway in mediating behavioral ethanol sensitivity, demonstrating roles for PKA (Thiele et al., 2000), adenylyl cyclase (Maas et al., 2005), and PACAP itself (Tanaka et al., 2004), thus validating the usefulness of *Drosophila* as a valuable tool for identifying candidate genes and pathways underlying the behavioral responses to ethanol.

In an effort to gain a better understanding of the neuroanatomical loci in which cAMP/PKA signaling might mediate ethanol sensitivity in *Drosophila*, Rodan et al. expressed an inhibitor of PKA signaling, UAS-PKA^{inh} (Li et al., 1995), in various subsets of the fly central nervous system using 59 different P[GAL4] driver lines (Rodan et al., 2002). Inhibition of cAMP/PKA signaling using most of these driver lines produces no effect on ethanol sensitivity, as measured using the inebriometer. However, three P[GAL4] lines: 201Y, c107, and c522, give increased resistance when crossed to UAS-PKA^{inh}. Rodan et al. focuses on one of these lines, 201Y, which shows a relatively restricted CNS expression pattern that includes the mushroom bodies, the dorsal giant interneurons (DGIs), ~13 neurons in the neurosecretory cells of the pars intercerebralis (PI), and 6 neurons in the ventral subesophageal ganglion (SEG). Chemical ablation of the mushroom bodies through larval feeding of hydroxyurea does not alter ethanol sensitivity in either wild-type flies or in flies expressing UAS-PKA^{inh} under the control of 201Y, suggesting that these structures, known to play crucial roles in olfactory and courtship conditioning (de Belle and Heisenberg, 1994; McBride et al., 1999) are

dispensable for the regulation of ethanol sensitivity. As three other P[GAL4] lines with DGI expression do not affect ethanol sensitivity in the inebriometer when driving UAS-*PKA*^{inh}, the authors suggest that the DGIs are also unlikely to be the neurons responsible for the altered behaviors seen in 201Y+ UAS-*PKA*^{inh} flies. Instead, the authors offer the intriguing possibility that a subset of the neurosecretory cells that make up the SEG and/or the PI may play a role in regulating ethanol sensitivity in *Drosophila*.

III. FasciclinII

As mutations in the learning and memory mutants *amnesiac* and *rutabaga* (*rut*) result in increased ethanol sensitivity in the inebriometer (Moore et al., 1998), Cheng et al. asked the question whether their *Drosophila fasciclinII* (*fasII*) mutants, which perform poorly after olfactory conditioning, would show altered ethanol sensitivity as well (Cheng et al., 2001). Indeed, both loss of function *fasII* mutants tested, *fasII* ^{rd1} and *fasII* ^{rd2}, show increased ethanol sensitivity in the inebriometer, suggesting a role not only for this cell adhesion receptor in mediating behavioral ethanol sensitivity, but also bringing up the tantalizing possibility that there may exist substantial overlap between the pathways that underlie learning and memory and ethanol sensitivity.

IV. Dopamine Regulation of Acute Ethanol Responses

The role of dopaminergic systems in mediating the responses to psychostimulants such as cocaine and methamphetamine, as well as to other drugs of abuse such as alcohol, has been the topic of study in mammalian systems for a number of years (Di Chiara, 1995; White, 1996). In an effort to better understand the neurotransmitter systems that

may regulate acute ethanol responses in *Drosophila*, Bainton et al. tested the effects of pharmacologically reducing dopamine levels in adult flies (Bainton et al., 2000) by feeding them 3-iodotyrosine (3IY), a competitive inhibitor of tyrosine hydroxylase, the enzyme that catalyzes the rate-limiting step in the synthesis of dopamine (Neckameyer, 1996). Interestingly, Bainton et al. found that when tested in the line crossing assay, flies fed 3IY show a marked and significant decrease in their levels of locomotor activation during the hyperactive phase compared to controls, an effect which can be reversed by simultaneously feeding the dopamine precursor, L-Dopa, along with the 3IY (Bainton et al., 2000). The sedation phase of 3IY treated flies is indistinguishable from that of controls. Ethanol pharmacokinetics are not altered in 3IY treated flies, as ethanol absorption levels at various time points are comparable between the various treatment groups. In addition to affecting ethanol induced behaviors, Bainton et al. found that pharmacologically reducing dopamine levels also blunts acute responses to both cocaine and nicotine, demonstrating a broad involvement of dopaminergic systems in regulating the responses to a variety of drugs of abuse in *Drosophila* (Bainton et al., 2000).

V. $GABA_B$ receptor, $GABA_BR1$

In mouse studies, the $GABA_B$ receptor has been implicated in mediating both acute behavioral responses to ethanol (Dar, 1996) as well as the development of ethanol tolerance (Zaleski et al., 2001). In a study by Dzitoyeva et al., the authors asked whether silencing a subtype of $GABA_B$ receptors in the fly, $GABA_BR1$, would affect duration of recovery to ethanol-induced sedation (Dzitoyeva et al., 2003). The authors found that injection of an RNAi directed against $GABA_BR1$, which knocks levels of endogenous

$GABA_BR1$ mRNA down to undetectable levels, reduces the duration of ethanol-induced immobility in an ethanol recovery assay. A similar attenuation of ethanol induced motor impairment is seen upon injection of the $GABA_B$ antagonist CGP 54626 (Dzitoyeva et al., 2003; Mezler et al., 2001). This study, confirming results seen previously in mice, demonstrates the utility of reverse genetic approaches such as RNAi in uncovering the molecules modulating behavioral responses to ethanol in *Drosophila*.

VI. Neuropeptide F (NPF) and its receptor, NPFR1

Mice lacking the signal molecule neuropeptide Y (NPY) or the NPY receptor Y1 show decreased sensitivity to ethanol-induced sedation and increased alcohol consumption, while NPY overexpressing mice display the opposite phenotype (Thiele et al., 1998; Thiele et al., 2002). Wen et al. tested whether the fly homolog of NPY, neuropeptide F (NPF), plays a similar role in mediating ethanol-induced behaviors in *Drosophila* (Wen et al., 2005). Ablation of NPF expressing cells and ablation of cells expressing the NPF receptor, NPFR1, through driving the expression of UAS-*DTI* (encoding an attenuated diphtheria toxin; Han et al., 2000) using either an *npf-gal4* or *npfr-gal4* driver line, both cause the flies to become resistant to ethanol induced sedation, as measured in a simple sedation assay. This decrease in ethanol sensitivity can also be elicited through acute silencing of NPF neurons and NPFR1 neurons during adulthood, 60 minutes before the sedation assay, using the UAS-*shibire*^{ts1} transgene, which encodes a temperature-sensitive allele of a semi-dominant negative form of dynamin (Kitamoto, 2002). Furthermore, overexpression of NPF in wild-type flies using the *386Y-gal4* line, which drives expression in brain cells, including peptidergic neurons (Taghert et al.,

2001), results in the opposite behavioral phenotype, i.e. increased sensitivity to ethanol-induced sedation. The effects seen do not appear to be due to alterations in ethanol pharmacokinetics, nor does disruption of NPF/NPFR1 signaling affect sedation responses to another sedative agent, diethyl ether. Taken together, these results support a role for NPF signaling in the modulation of ethanol sensitivity, and highlight another parallel between mammalian and fly models for alcohol research.

VII. *moody*: a gene encoding two novel GPCRs

In a forward genetic screen for *Drosophila* mutants with altered sensitivity to cocaine, Bainton et al. identified and characterized mutants in gene CG4322, which they named *moody* (Bainton et al., 2005). When testing *moody* flies' responses to other drugs of abuse, the authors found that while *moody* mutants show increased sensitivity to nicotine, they also show significantly decreased sensitivity to ethanol, measured as an increased mean elution time in the inebriometer. Ethanol absorption assays ruled out altered ethanol pharmacokinetics as being responsible for this phenotype. While the function of *moody*, which encodes two novel G protein-coupled receptors (GPCRs), is not yet fully elucidated, Bainton et al. showed that both Moody isoforms are coexpressed in glia that surround the central nervous system and are required for the maintenance of normal blood-brain barrier permeability. The authors provide evidence that the drug-induced behavioral phenotypes observed in *moody* flies are not simply caused by altered accessibility of the drugs to the central nervous system, suggesting a more complex role for *moody* in regulating drug-induced (including ethanol-induced) behaviors in *Drosophila*.

VIII. *white rabbit / RhoGAP18B*

In a forward genetic screen for *Drosophila* mutants with altered sensitivity to nicotine, Rothenfluh et al. isolated three mutants in the gene *RhoGAP18B*, dubbed *white rabbit*, which all show resistance to the development of nicotine-induced behaviors (Rothenfluh et al., 2006). Further characterizations of these and other loss of function mutants in the *white rabbit /RhoGAP18B* gene region revealed that *white rabbit* mutants are also strongly resistant to ethanol induced sedation, as measured in both the booz-o-mat automated motion tracking system as well in the loss of righting (LOR) sedation assay. As other GTPase-activating proteins (GAPs) in the Rho family have been shown to act as molecular switches, regulating the activity of Rho GTPases and changes in the actin cytoskeleton (Etienne-Manneville and Hall, 2002), Rothenfluh et al. tested and found *in vitro* that RhoGAP18B can indeed stimulate GTPase activity, and that expression of transgenes encoding dominant-negative or constitutively active versions of various Rho-type GTPases can enhance or decrease ethanol sensitivity, respectively. As the *white rabbit / RhoGAP18B* locus encodes several transcripts, RA through RD, the authors tested how manipulations of specific *white rabbit / RhoGAP18B* transcript levels would affect ethanol induced behaviors. Interestingly, they found that while the RC transcript mediates sensitivity to ethanol-induced sedation, the RA transcript mediates ethanol-induced hyperactivity. Thus, two different RhoGAPs, encoded by the same gene, appear to regulate different aspects of the ethanol response: hyperactivity and sedation.

IX. *homer*

In a microarray experiment to identify genes exhibiting a change in expression in fly heads upon a single and/or several treatments of ethanol, Urizar et al. isolated *homer*, a gene with mildly (17% and 24%) decreased expression in response to single or multiple ethanol exposures, respectively (Urizar et al., 2007). The *in vivo* role of Homer is yet unknown, although its interactions with various synaptic proteins such as the group 1 metabotropic glutamate receptors (mGluR1/5) suggest that it may play a role in synaptic structure maintenance and/or plasticity (Tu et al., 1998; Xiao et al., 1998). When tested in a sedation assay, it was found that *homer*^{R102} null mutant flies sedate approximately 5 minutes earlier than controls, a difference that was not due to altered ethanol pharmacokinetics. The sedation sensitivity phenotype of *homer* mutant flies can be partially rescued by pan-neuronal expression of a wild-type UAS-*homer* transgene using *elav* ^{c155}*-gal4* or by using two other drivers, *c819-gal4* and *c42-gal4*, which drive expression, among other places, in the R2 and R4 neurons of the ellipsoid body in the fly brain. Their data suggest that the ellipsoid body, a component of the central complex which has been implicated in the maintenance of various aspects of locomotor activity (Martin et al., 1999), may play an important role in mediating some of the behavioral effects of ethanol intoxication in fruit flies. The finding that loss of function of *homer* results in increased sensitivity to ethanol induced sedation supports the results of previous studies of *homer2* mutant mice, which are also more sensitive to ethanol-induced sedation and fail to develop place preference or locomotor sensitization to multiple presentations of ethanol (Szumlinski et al., 2003, 2005).

Functional Ethanol Tolerance: Rapid and Chronic

i.) Rapid Tolerance: Tolerance induced by a single short pre-exposure to a relatively high concentration of ethanol

While the studies described above detail the use of *Drosophila* in studying the effects of a single ethanol exposure on behavior, other studies have focused on the effects of multiple ethanol exposures on fly intoxication, specifically the development of functional ethanol tolerance, which can be defined as an acquired resistance to the intoxicating effects of ethanol. Studies of ethanol tolerance in *Drosophila* were pioneered by Scholz et al., who discovered that multiple exposures to ethanol lead to the development of functional ethanol tolerance, manifested as an increased mean elution time (MET) in the inebriometer (Scholz et al., 2000). The protocol for inducing tolerance in this study involves running flies through the inebriometer once, letting them recover in non-ethanol containing food vials at 25°C, then running them back through the inebriometer four hours after the initial exposure, a protocol which induces a 34% increase in resistance to ethanol-induced loss of postural control. This tolerance is functional rather than metabolic, as tolerance development is not associated with changes in ethanol absorption or metabolism. Scholz et al. went on to show that the development of functional rapid tolerance requires the structural and functional integrity of central brain regions, with the data especially suggestive for a role of central complex brain regions such as the ellipsoid body. Finally, Scholz et al. showed that $T\beta H^{nM18}$ mutant flies, which lack the neuromodulator octopamine (Monastirioti et al., 1996), the fly

analog of noradrenaline, are also impaired in tolerance development, supporting findings in mice which had demonstrated a requirement for brain noradrenergic systems for the development of functional ethanol tolerance (Tabakoff and Ritzmann, 1977).

Methods for Inducing and Assaying Rapid Tolerance in *Drosophila*

In addition to the method described above by Scholz et al. to induce rapid tolerance, i.e. running the same set of flies through the inebriometer twice with a 4 hour rest period between runs, other groups have devised their own methods for eliciting and quantifying rapid tolerance, that is, tolerance elicited by a single pre-exposure to ethanol. In Godenschwege et al., initial exposure involves exposing 20 four-day-old male flies to 50% ethanol vapor in perforated 50 ml Falcon tubes until the flies are sedated, letting them recover for 4 hours, then exposing them again to 50% ethanol vapors in the same apparatus and counting numbers of sedated flies during both the 1st and 2nd exposures (Godenschwege et al., 2004). In Berger et al., flies are exposed in parallel to either a 60/40 ethanol/air mixture in perforated tubes for 30 minutes or to humidified air alone for 30 minutes, given 3 hours and 30 minutes to recover in food vials at 25°C, then tested in a recovery assay (Berger et al., 2004). Tolerance is calculated as the difference in mean recovery time (MRT) between the ethanol-pre-exposed and the air-pre-exposured flies. Similarly, the protocol in Cowmeadow et al. involves exposing parallel groups of flies to either ethanol vapors or humidified air in an "inebriator" (see Section VII. above for description) until all the flies in the ethanol chamber are sedated (Cowmeadow et al., 2005). Flies are then allowed to recover in food vials, and then later both sets of flies are returned to the inebriator and sedated with ethanol vapors. Tolerance is quantified by

comparing the recovery curves for flies receiving their second ethanol exposure versus those receiving their first ethanol exposure.

Genes and Pathways Involved in Mediating Rapid Tolerance to Ethanol in *Drosophila*

Several studies have shed light onto some of the molecules and pathways that mediate rapid tolerance to ethanol in *Drosophila*. In addition to the studies detailed in the following sections, it is interesting to note that in some cases, the same gene appears to regulate both acute sensitivity to ethanol intoxication as well as development of rapid tolerance. *Homer* mutant flies, for example, which exhibit increased sensitivity to ethanol-induced sedation (see IX. above), are also defective in the development of rapid tolerance, an impairment that can be rescued through spatially restricted expression of a wild-type UAS-*homer* transgene in cells including the R2 and R4 neurons of the ellipsoid body (Urizar et al., 2007). In addition, $GABA_BR1$, which mediates sensitivity to ethanol-induced sedation as measured in a recovery assay (see Section V. above), also appears to regulate rapid tolerance development: pretreatment with the $GABA_B$ agonist 3-APMPA effectively blocks rapid tolerance to ethanol (Dzitoyeva et al., 2003). These studies show that some of the same molecules can regulate different aspects of the ethanol response: acute sensitivity and rapid tolerance.

a.) *hangover*

In a forward genetic screen to identify genes and pathways involved in mediating rapid tolerance development in *Drosophila*, Scholz et al. isolated a null mutant, AE10, in the gene CG32575 which displays normal initial inebriometer sensitivity to ethanol but

shows a reduced ability to develop rapid tolerance when exposed to ethanol a second time in the inebriometer (Scholz et al., 2005). The gene CG32575, renamed *hangover* (*hang*), encodes a large, zinc finger-containing protein with a calcium-binding EF hand, suggesting that the Hangover protein may play a role in nucleic acid binding and may be regulated by calcium. When tested for responses towards environmental stressors, it was found that $hang^{AE10}$ flies show increased sensitivity to paraquat-induced oxidative-stress and have an overall decreased lifespan. Scholz et al. also found that a different form of stress, a heat shock exposure of 37°C for 30 minutes, could mimic the effects of ethanol pre-exposure and lead to an increased mean elution time when wild-type heat-shocked flies were tested in the inebriometer 4 hours later. $hang^{AE10}$ flies also show defects in development of this heat-ethanol cross-tolerance, suggesting an overlap between the cellular changes induced by heat and ethanol in *Drosophila*.

b.) *slowpoke*

The *slowpoke* gene encodes a large-conductance calcium-activated potassium channel, also known as the BK channel, which has been shown to affect action potential shape, neuronal excitability, and neurotransmitter release (Gribkoff et al., 2001). In *C. elegans*, *slowpoke* was identified in a screen for genes regulating ethanol-induced behaviors: mutations in this BK channel gene cause a decrease in ethanol sensitivity (Davies et al., 2003). Cowmeadow et al., endeavoring to determine if the same would hold true in *Drosophila*, found that the null *slowpoke* mutant, slo^4, shows practically no development of rapid tolerance, as measured in the inebriator (Cowmeadow et al., 2005). The authors showed that this impairment is not due to altered ethanol pharmacokinetics,

and also describe that various loss-of-function mutants in other ion channel genes, including the *Shaker* potassium channel gene and the *paralytic* voltage-activated sodium channel gene, show normal tolerance development. In a later study, Cowmeadow et al. went on to show that levels of neuronally expressed, but not muscular/tracheally expressed, *slowpoke* are induced 6 hours after ethanol-induced sedation (Cowmeadow et al., 2006). In addition, the authors show that heat-shocked induced expression of *slowpoke* in a *slowpoke* mutant background results in faster recovery to ethanol-induced sedation, lending further support to the role of this gene in mediating ethanol-induced behaviors, including development of rapid tolerance (Cowmeadow et al., 2006).

c.) *synapsin*

In a study examining the various morphological and behavioral phenotypes associated with a null mutation in the single fly *synapsin* gene, which codes for a synaptic vesicle associated phosphoprotein, Godenschwege et al. test their Syn^{97} null mutant flies for both acute ethanol sensitivity as well as for development of rapid tolerance in a sedation assay, using 50 ml Falcon tubes into which flies are introduced and ethanol vapors circulated (Godenschwege et al., 2004). While Syn^{97} flies behave similarly to controls in the acute ethanol sensitivity assay, they show a modest, but significant, increase in tolerance development compared to controls. Interestingly, in their study, Godenschwege et al. find that their Syn^{97} flies are relatively normal otherwise in many of their other assays, with no obvious defects in brain morphology or striking alterations in general behavior, suggesting that at least in *Drosophila*, Synapsin may modulate more subtle effects in nervous system function.

ii.) Chronic Tolerance: Tolerance induced by a prolonged pre-exposure to a relatively low concentration of ethanol

In contrast to rapid tolerance, which develops after a single exposure to a relatively high concentration of ethanol, Berger et al. found that *Drosophila* also develop chronic tolerance, i.e. tolerance resulting from prolonged exposure to a relatively low concentration of ethanol (Berger et al., 2004). Perhaps to gain a better understanding of the physiological or behavioral consequences of chronic, low-level drinking in humans, Berger et al. subjected flies, kept in food-containing vials, to a continuous alcohol vapor pre-exposure (at a relatively low concentration of 10/80 ethanol/air mixture) lasting from 4 hours up to 2 days, a paradigm which results in low but detectable levels of internal ethanol accumulation after each pre-exposure. Tolerance development is then measured through either a recovery assay or by putting the flies through the inebriometer. Indeed, in comparison to flies that had received a mock pre-exposure to humidified air only, ethanol pre-exposed flies show robust chronic tolerance in all treatment conditions, with % tolerance ranging from 12% for flies that received a 4 hour ethanol pre-treatment to 31% for flies that received a 48 hour pretreatment. Several differences between chronic tolerance and rapid tolerance were elucidated by Berger et al.. Firstly, chronic tolerance appears to dissipate later than rapid tolerance: rapid tolerance completely dissipates 48 hours after the initial ethanol pre-exposure, whereas chronic tolerance is still present after 48 hours (but not after 72 hours). Secondly, treatment with the protein synthesis inhibitor cycloheximide (CMX) eliminates the development of chronic, but not rapid, tolerance. Finally, $T\beta H^{nM18}$ mutant flies, which lack the neuromodulator octopamine (Monastirioti et

al., 1996), the fly analog of noradrenaline, show decreased development of rapid tolerance (as described above), but not in chronic tolerance. Thus, rapid and chronic tolerance represent two distinguishable and dissociable forms of ethanol tolerance in *Drosophila*.

Ethanol Preference Assays

Given that fruit flies spend much of their lives in the company of fermenting fruit, it is somewhat surprising the dearth of literature that is available describing ethanol preference assays in *Drosophila*. One study aimed to determine how two alcohol dehydrogenase (*Adh*) fly strains, differing in their efficiencies at metabolizing ethanol, differ in female oviposition preference and larval preference behaviors when presented which a choice between media with or without ethanol (Cavener, 1979). Cavener found that females of both *Adh* fly strains show a similar degree of minor aversion towards depositing their eggs on food supplemented with 10% ethanol. When examining larval behavior, however, Cavener found that while *Adh SS* larvae (which have lower ADH activity) show no preference between ethanol and non-ethanol supplemented agar, *Adh FF* larvae, which have approximately twice the ADH activity as *Adh SS* larvae and are thus more efficient at metabolizing ethanol as a potential food source, show a strong preference for agar supplemented with 17% ethanol over non-ethanol containing agar.

Cadieu et al. conducted a study using adult male *Drosophila* (of the fast ethanol metabolizing *Adh F* strain) to study ethanol preference in naïve flies as well as in flies that had previously been exposed to ethanol-supplemented food (Cadieu et al., 1999). They found that while ethanol naïve flies show a slight preference for media containing

3% ethanol over non-ethanol containing media, as measured by total time spent extending the proboscis into each medium, flies that were given a prior exposure to 3% ethanol for 24 hours prior to testing show a much stronger preference for the ethanol-supplemented food. Flies that were given a 24 hour pre-exposure to ethanol-supplemented food that also contained the ADH inhibitor 4-methylpyrazole (4-MP), however, show a strong avoidance of ethanol-containing food when placed in the ethanol preference assay, supporting the hypothesis that ability to efficiently metabolize ethanol has a strong influence on ethanol preference behaviors (Cadieu et al., 1999; Cavener, 1979).

C.) *Drosophila* as a Model System for Cocaine Research

Through the years, the highly addictive psychostimulant cocaine and its potential molecular targets have been the subjects of much research. In the synapse, cocaine potently inhibits the reuptake of various monoaminergic neurotransmitters through its interaction with various membrane bound monoamine transporters, including the dopamine transporter (DAT), the serotonin transporter (SERT), and the norepinephrine transporter (NET) (Ritz et al., 1990). While pharmacological studies using antidepressant drugs suggested that the dopamine transporter was the relevant molecular target mediating the reinforcing properties of cocaine (Tzschentke, 1998), more recent studies using DAT knockout mice have shown that the mechanisms underlying cocaine addiction are more complex. While DAT -/- mice lacking the dopamine transporter are indifferent to the locomotor stimulating effects of acute cocaine administration (Giros et al., 1996), they surprisingly still develop cocaine-conditioned place preference (Sora et al., 1998)

and still self-administer cocaine in a cocaine reward paradigm (Rocha et al., 1998), suggesting that cocaine is still rewarding to these animals that lack DAT. In addition, serotonin-transporter knockout mice also are able to establish cocaine-conditioned place preferences (Sora et al., 1998), while norepinephrine transporter knockout mice surprisingly show increased sensitivity to cocaine induced locomotor stimulation (Xu et al., 2000). Interestingly, double knockout mice lacking both DAT and SERT display no cocaine-conditioned place preference, suggesting that cocaine can mediate its rewarding actions through either the dopamine or the serotonin transporter, and that either transporter alone can compensate for the absence of the other in a single knockout mouse (Sora et al., 2001).

The fruit fly, *Drosophila melanogaster*, has proven to be a useful model organism cocaine research. In this section I will describe some of the assays used to quantify the behaviors elicited by acute and repeated cocaine administration, and describe how these assays have been utilized to identify some of the molecular and neuroanatomical substrates underlying cocaine sensitivity and sensitization.

Methods for Assaying the Acute Effects of Cocaine on *Drosophila* Behavior

One of the first descriptions of the array of cocaine-induced behavioral responses in *Drosophila* was reported by McClung and Hirsch, who delivered free-base cocaine volatilized from a heated filament to fruit flies for 1 minute, then transferred the flies to a glass chamber for behavioral scoring (McClung and Hirsh, 1998). Flies react to cocaine exposure in a dose dependent manner: low cocaine doses elicit increased grooming, stereotyped locomotion, and extension of the proboscis, medium cocaine doses elicit

rapid circling, backwards walking, and wing buzzing, while high cocaine doses elicit severe whole body tremor, loss of posture, and death. Behavioral scores are assigned by a trained observer using a scale from 0 to 7, where 0 represents normal behavior and 7 is assigned to the most severely affected flies. McClung and Hirsh found that repeated cocaine exposures leads to cocaine sensitization, and also found that a sexual dimorphism exists in the flies' responsiveness to cocaine: males are more severely affected than females (McClung and Hirsh, 1998).

In a second assay developed by Bainton et al., flies are exposed to volatilized cocaine then transferred into a narrow glass cylinder, the crackometer, lined with nylon mesh (Bainton et al., 2000). Flies are then knocked down to the bottom of the crackometer and the proportion of flies that fail to climb off the bottom of the cylinder are scored. Mock-treated flies display robust negative geotaxis and quickly climb to the top of the cylinder, while cocaine treated flies display defects in negative geotaxis in a dose dependent manner. In addition to using the crackometer assay, Bainton et al. also recorded the locomotor behavior of cocaine-treated flies in an automated locomotor tracking assay and quantified dose dependent decreases in locomotion and increases in circling behavior (Bainton et al, 2000).

In the past three years, several alternative methods for cocaine delivery and cocaine behavioral analysis have been described. In a departure from the traditional delivery of cocaine through volatalization, Dimitrijevic et al. found that dose-dependent cocaine behaviors and sensitization can be induced by intra-abdominal cocaine injections (Dimitrijevic et al., 2004). Individual cocaine treated flies are loaded into small glass tubes connected to the *Drosophila* Activity Monitoring System (Trikinetics, Waltham,

MA, USA) and locomotor activity is detected and counted by infrared light beams. As a third method of drug delivery, Lease and Hirsh described cocaine delivery to cold-anesthetized flies through the use of a modified graphic arts airbrush (Lease and Hirsh, 2005). After being "airbrushed" with cocaine, the flies are dried off using an airstream, then transferred to a glass viewing chamber and scored for cocaine-induced behaviors using the 0-7 scoring system described above. Lastly, George et al. described a "bottom-counting" video system wherein cocaine exposed *Drosophila* are transferred to glass vials positioned over an upward-pointing camera (George et al., 2005). The camera, which is focused only on the very bottom of the vial, picks up only those flies that remain on the vial bottom post cocaine exposure, presumably due to an impaired ability to display normal negative geotaxis behavior, giving the observer an relatively easy way to quantitatively measure sensitivity to acute cocaine exposure in *Drosophila*.

Genes and Biochemical Pathways Mediating Cocaine Sensitivity in *Drosophila*

As in mammals, aminergic neurotransmitter systems have been the target of several studies aimed at understanding the molecular pathways underlying cocaine sensitivity in *Drosophila*. Like mammals, *Drosophila* utilize both dopamine (DA) and serotonin (5-hydroxytryptamine; 5HT) as neurotransmitters (Buchner, 1991) and the distribution of catecholamine-containing neurons, including dopaminergic neurons, has been well characterized (Budnik and White, 1988). In addition, both a cocaine-sensitive dopamine transporter (dDAT) and a cocaine-sensitive serotonin transporter (dSERT1) have been identified in flies (Porzgen et al., 2001; Corey et al., 1994). Evidence for the role of catecholamines in regulating behavioral sensitivity to cocaine in *Drosophila* was

first described by Bainton et al., where it was found that flies with reduced dopamine levels (induced by feeding the flies 3-iodotyrosine, a competitive antagonist of the enzyme tyrosine hydroxylase) show a significant reduction in their sensitivity to the effects of cocaine, as measured in the crackometer (Bainton et al., 2000). Treatment with L-Dopa, a precursor to dopamine synthesis, was sufficient to restore normal cocaine sensitivity to these 3-iodotyrosine treated flies, confirming that the effects seen were due to decreased dopamine levels. Concurrently, a role for dopamine and serotonin neurons was demonstrated by Li et al., who found that ectopic G-protein expression of either a stimulatory or inhibitory Gα subunit, or expression of tetanus toxin light chain in dopamineric and serotonergic neurons blocks the development of cocaine sensitization (Li et al., 2000). A more recent study has also tied in cocaine sensitivity to monoaminergic neurotransmitter systems in *Drosophila* by demonstrating that flies overexpressing the vesicular monoamine transporter (DVMAT-A) show decreased cocaine sensitivity as measured in the crackometer, presumably due to a homeostatic change in synaptic machinery modifying the flies responses to cocaine (Chang et al., 2006). Finally, the trace biogenic amine tyramine, a precursor to the fly neurotransmitter octopamine, has also been shown to play a role in cocaine sensitization, as *inactive* mutant flies that have reduced amounts of tyramine in the brain fail to sensitize to repeated cocaine exposures (McClung and Hirsh, 1999).

In addition to aminergic neurotransmitter systems, studies of cocaine sensitivity in *Drosophila* have also revealed a novel role for circadian genes in regulating cocaine sensitization. Various strains of flies carrying loss of function mutations in different circadian genes, such as *period*, *clock*, *cycle*, and *doubletime*, show an inability to

develop cocaine sensitization (Andretic et al., 1999). Importantly, subsequent studies in mice have confirmed the influence of circadian genes on cocaine sensitization (Abarca et al., 2002; Uz et al., 2003), validating the usefulness of *Drosophila* in studying the mechanisms underlying the behavioral responses to cocaine.

Forward genetic screens for *Drosophila* mutants with altered acute responses to cocaine have yielded interesting results. In one study, it was found that loss of function mutations in *moody*, a gene which encodes two orphan G protein-coupled receptors (GPCRs) involved in the maintenance of the integrity of the blood-brain barrier, show increased sensitivity to cocaine in the crackometer assay (Bainton et al., 2005). Another study identified the *Drosophila* LIM-only (*Lmo*) gene, encoding a regulator of LIM-homeodomain proteins, as mediating cocaine sensitivity in the crackometer (Tsai et al., 2004). Interestingly, the latter study highlighted a novel role for a specific group of neurons, the PDF-expressing ventral lateral neurons which function as the principal pacemaker cells in *Drosophila* (Helfrich-Forster, 1997; Renn et al., 1999), in regulating sensitivity to cocaine-induced behaviors, providing insight into the neuroanatomical pathways that underlie responses to this powerfully addictive psychostimulant drug.

Part III

A.) The Insulin/Insulin-like Growth Factor Signaling (IIS) Pathway:

Overview and Insights

Overview

Known to most studious Introductory Biology freshmen cramming for their endocrinology mid-terms as the hormone which opposes the action of glucagon to lower blood sugar levels, in part by slowing glycogen breakdown in the liver and stimulating cells of various tissues, including muscle, to take up glucose from the blood (Campbell, 1993), the insulin (and the insulin-like growth factor signaling pathway) has also been shown to regulate a host of other crucial life-processes. In this section, I will introduce the IIS pathway in mammals and the invertebrate model organisms *Drosophila melanogaster* and *Caenorhabditis elegans* (*C. elegans*), and describe how recent studies in these various systems have revealed how the IIS pathway regulates longevity, cell growth, cell size, and central nervous system mediated feeding behaviors.

The cascade of cellular events that occurs upon binding of insulin or insulin-like growth factor-I (IGF-I) to the extraceullar α-subunits of their respective receptor tyrosine kinases has been well described (reviewed in Kido et al., 2001; Lodish et al., 2000) and is diagrammed in the accompanying Introductory Figure 1 (see Introductory Figure 1 below from Garofalo, 2002). In mammals, substrate binding to the insulin / IGF-1 receptors results in receptor autophosphorylation of the receptors' β-subunit's intracellular domain. The now activated insulin / IGF-1 receptors then phosphorylate and activate insulin receptor substrates (IRSs), which in turn bind to and recruit to the membrane the adaptor

p85 subunit of phosphatidylinositol 3-kinase (PI3K) via their interactions with the SH2 domains of p85. This then causes the recruitment of the catalytic subunit of PI3K, p110, to the membrane, leading to the phosphorylation of phosphatidylinositol-4,5-P2 (PIP2), generating phosphatidylinositol-3,4,5-P3 (PIP3). PIP3 can bind to, via pleckstrin homology (PH) domains, and activate various downstream protein targets, including the cytoplasmic protein kinases PDK1 (phosphoinositide-dependent protein kinase 1) and AKT/PKB (protein kinase B), which can then in turn phosphorylate and regulate the activities of various effectors, including forkhead-related transcription factors (FKHR), GSK-3 (glycogen synthase kinase-3), and S6K (p70 ribosomal S6 kinase). The IIS pathway is negatively regulated by PTEN (phosphatidylinositol 3,4,5,-triphosphate 3-phosphatase), which catalyzes the conversion of PIP3 back to PIP2. It is worthy to note that the IIS pathway can also signal via a Ras-dependent pathway, activating MAP kinase cascades through the interaction of phosphorylated IRS with the SH2 domain of the adaptor protein GRB2, which in turns binds to Sos protein, leading to the conversion of Ras-GDP to Ras-GTP and the activation of downstream MAP kinase effector molecules.

The various components of the IIS pathway have been remarkably conserved across various species, including *C. elegans* and *Drosophila melanogaster* (See Introductory Figure 1 below from Garofalo, 2002). *Drosophila*, for example, expresses seven insulin-like genes (*dilps*) (Cao and Brown; 2001; Brogiolo et al., 2001), several of which are expressed in a small (7 cell) cluster of neurosecretory cells in the pars intercerebralis region of the fly brain (Ikeya et al., 2002). A single insulin receptor homolog, dInR (Fernandez-Almonacid and Rosen, 1987), an IRS (CHICO) (Bohni et al., 1999), catalytic (Leevers et al., 1996) and adaptor (Weinkove et al., 1999) subunits of

PI3K (p110 and p60, respectively), as well as homologs of all the other major downstream IIS components have been identified and characterized in *Drosophila* (reviewed in Garofalo, 2002). In the following sections I will describe how studies of IIS pathways in various model organisms, specifically *Drosophila melanogaster*, *C. elegans*, and *Mus musculus* (mouse), have yielded exciting insights into the various life processes regulated by this ancient and conserved pathway.

Insulin/insulin-like growth factor signaling (IIS) pathway regulation of lifespan

The tiny but mighty nematode *Caenorhabditis elegans* (*C. elegans*) has yielded great insight into how hormonal regulation, especially through the insulin/insulin-like growth factor signaling (IIS) pathway, can profoundly influence lifespan. It all began with a study which found that mutations in the gene *daf-2*, which encodes a homolog of the insulin and insulin-like growth factor-I (IGF-I) receptors (Kimura et al., 1997), causes *C. elegans* adults to live more than twice as long as wild-type worms (Kenyon et al., 1993). This lengthened lifespan is dependent on the downstream activity of a second gene, *daf-16*, which encodes a transcription factor in the HNF-3/forkhead family (Kenyon et al., 1993; Lin et al., 1997; Ogg et al., 1997). It was found that *daf-2* functions cell nonautonomously to regulate lifespan (Apfeld and Kenyon, 1998). Furthermore, it was found that this effect is due to *daf-2*/*daf-16* pathway signaling during adulthood, as RNA interference (RNAi) of *daf-2* and *daf-16* specifically during adulthood is sufficient to alter lifespan (Dillen et al., 2002). In addition to *daf-2* and *daf-16*, several other genes in the insulin signaling cascade have been implicated in the regulation of lifespan, including *age-1*, which encodes a PI3K (Friedman and Johnson, 1988; Morris et al.,

1996), *pdk-1*, which encodes a PDK1 homolog (Paradis et al., 1999), *akt*, which encodes protein kinase B (Paradis et al., 1998), and two genes encoding insulin/IGF-I-like homologs: *Ceinsulin-1* (Kawano et al., 2000) and *ins-7* (Murphy et al., 2003). Various studies have aimed to pinpoint which tissues are important for the IIS pathway regulation of lifespan, with one study offering evidence for the nervous system (Wolkow et al., 2000) and another for the intestinal system (Libina et al., 2003) as being key regulators of lifespan. Interestingly, various other manipulations which extend lifespan in *C. elegans*, such removal of proliferating germ cells (Hsin and Kenyon, 1999; Berman and Kenyon, 2006), ablation of certain gustatory neurons (Alcedo and Kenyon, 2004), and mutation of various proteasomal E3 ligase genes (Ghazi et al., 2007), also extend lifespan, at least in part, through the IIS pathway, offering insight into some of the inputs and outputs through which this pathway may regulate longevity.

The role of the IIS pathway in regulating lifespan has been upheld in the fruit fly, *Drosophila melanogaster*, as well. Hypomorphic mutations in the *insulin-like receptor* (*InR*) gene cause females to live 85% longer than wild-type controls (Tatar et al., 2001). As juvenile hormone (JH) synthesis is reduced in *InR* mutants, and treatment with JH is sufficient to rescue wild type longevity to *InR* mutants, the authors suggest that JH deficiency, resulting from *InR* mutations, underlies the extended longevity observed in *InR* mutants (Tatar et al., 2001). Concurrently, it was found that a null mutation in the insulin receptor substrate homolog *chico* significantly increases median lifespan by up to 48% in homozygous mutants and 36% in heterozygous mutants (Clancy et al., 2001). In both these studies the effect on longevity is much more pronounced in females than in males, for reasons unknown. More recently, two groups reported that overexpression of

dFOXO, the fly ortholog of mammalian FOXO3a and DAF-16 in *C. elegans*, is sufficient to increase lifespan of both males and females when expressed in the adult head fat body, the fly tissue that carries out functions equivalent to those of the mammalian liver (Hwangbo et al., 2004; Giannakou et al., 2004). Interestingly, it was found that activated dFOXO expression in the adult head fat body causes a concurrent decrease in mRNA levels of *dilp-2*, a *Drosophila* insulin-like peptide which is expressed specifically in the pars intercerebralis (PI) neurosecretory cells of the fly brain (Rulifson et al., 2002), whereas *dilp-3* and *dilp-5* expresssion levels in the PI remained unchanged, suggesting a specific cell non-autonomous feedback loop in the IIS pathway that may function to control aging in the fruit fly (Hwangbo et al., 2004).

In mammals, evidence for the involvement of the IIS pathway in regulating longevity has been found in mice, where heterozygous knockout mice in the insulin-like growth factor type 1 receptor (*Igf1r*) gene live on average 26% longer than wild-type controls (homozygous *Igf1r* mutants are lethal) (Holzenberger et al., 2003). These *Igf1r* +/- mice are also resistant to oxidative stress, a determinant of aging (Holzenberger et al., 2003). Furthermore, mice overexpressing the hormone Klutho, which inhibits IGF-1 signaling by inhibiting FOXO phosphorylation, are also long lived, with an average increase in lifespan of 22% over controls (Kuruso et al., 2005). In another study, it was found that fat-specific insulin receptor knockout (FIRKO) mice show an increase in lifespan of 18% over controls, an effect that is seen in both males and females (Bluher et al., 2003). Mutations that affect expression of upstream genes whose products stimulate insulin/IGF-1 production, for example mutants in the growth hormone receptor, can also lead to increased longevity (Coschigano et al., 2003), and in a recent study using IRS2

heterozygote knockout mice, it was found that decreasing IRS2 signaling either pan-organismically or just in the brain is sufficient to extend life span up to 18% (Taguchi et al., 2007). It will be interesting to see how closely future studies in mammals will parallel those results previously seen in *C. elegans* and *Drosophila*.

Insulin/insulin-like growth factor signaling (IIS) pathway regulation of growth

Studies in *Drosophila* have revealed that manipulations of practically any of the canonical IIS pathway components can result in alterations in cell growth, cell size, organismal size, and/or organismal developmental time. Reducing insulin signaling by ablating the insulin producing cells (IPCs) of the brain, for example, results in larvae that are developmentally delayed and causes decreased body size in both larvae and adults (Rulifson et al., 2002). Conversely, overexpression of the insulin-like genes (*dilps*) normally expressed in the IPCs results in increased body size, and interestingly, expression of two of these *dilps*, *dilp3* and *dilp5*, appear to be regulated by nutrient availability (Ikeya et al., 2002).

While many loss-of-function mutations in the insulin receptor (InR) in *Drosophila* are lethal, loss-of-function mutations that are viable have been shown to result in severe (10 day) developmental delays and growth deficiencies in both larvae and adults (adult body weight reduced to 56% that of controls) (Chen et al., 1996). Closer examination of organs, such as the eye and the wing, revealed that these growth deficiencies are due to cell autonomous reductions in both cell size and cell number (Brogiolo et al., 2001). Accordingly, mutants homozygous for *chico*, which encodes the *Drosophila* insulin

receptor substrate (IRS), also are drastically smaller in body size compared to controls, again due to a reduction in both cell number and cell size (Bohni et al., 1999).

Studies of the various IIS pathway component mutants downstream of InR and IRS have yielded similar growth deficiency phenotypes. Overexpression of the PI3K catalytic subunit, Dp110, in wing or eye imaginal discs gives rise to adult flies with enlarged wings or eyes, respectively (Leevers et al., 1996), while inhibiting Dp110 signaling in these tissues results in smaller wings and eyes (Leevers et al., 1996; Weinkove et al., 1999). Overexpression of one of the downstream targets of PI3K signaling, PDK-1, results in a PI3K dependent increase in cell and organ size (Cho et al., 2001; Rintelen et al., 2001), while overexpression of a negative regulator of IIS pathway signaling, DPTEN, results in a decrease in cell size and number, as measured in the eye and wing (Goberdhan et al., 1999). Interestingly, manipulations of IIS pathway components downstream of PDK-1 appear to affect cell size or cell number, but not both. For example, tissue specific perturbation of Akt/PKB signaling, resulting in increased or decreased Dakt1 activity, positivitely or negatively affect cell size in the eye and the wing, respectively, without appearing to affect cell number (Verdu et al., 1999). This was also seen in mutants deficient in the S6 kinase gene, which have decreased body size due to a decrease in cell size but not cell number (Montagne et al., 1999; Radimerski et al., 2002). In the case of the forkhead-related transcription factor FOXO, whose activity is negatively regulated by the IIS pathway, the reasons underlying the growth phenotypes of dFoxo mutant flies is somewhat controversial. While one group contends that the reduction in organ size seen when overexpressing dFOXO is due to decreases in both cell size and cell number (Kramer et al., 2003), two other groups offer evidence that dFOXO

regulates organ size by specifying cell number but not cell size (Puig et al., 2003; Junger et al., 2003).

In mice, roles for the IIS pathway in regulating cell growth and survival have been shown in various *in vitro* studies: in freshly dissociated neuroblasts, for example, IGF-1 stimulates cell division and DNA synthesis (DiCicco and Black, 1988). *In vivo*, newborn *Igf-1* -/- mice exhibit a strong growth deficiency (60% of normal birthweight) while mice that are null for the Igf-1 receptor (*Igf1r* -/-) exhibit an even stronger growth deficiency (45% normal size) and die at birth of respiratory failure (Liu et al., 1993). Examination of CNS tissue show that homozygous *Igf1* -/- mice have strongly reduced brain and spinal cord size, due at least in part to decreased cell number (Beck et al., 1995), whereas transgenic mice overexpressing IGF-1 have larger brain sizes (Carson et al., 1993). Similarly, *Igf-II* +/- mice also have a growth phenotype, with these heterozygotes having approximately 60% of the body weight as their wild-type littermates (DeChiara et al., 1990). Similarly, *Irs-1* -/- mice homozygous for targeted disruption of the insulin receptor substrate IRS-1 are viable and fertile but show retarded growth, with body weight about 70% that of wild-type littermates at 3, 8, and 15 weeks of age (Tamemoto et al., 1994). Interestingly, a role of the IIS pathway in growth regulation has also been found in humans, as it was reported that a homozygous missense mutation in the kinase domain of the insulin receptor in a human patient resulted in intrauterine growth retardation and leprechaunism, as well as severe insulin resistance (Takahashi et al., 1997).

Functions of Insulin in the Central Nervous System / Regulation of Feeding

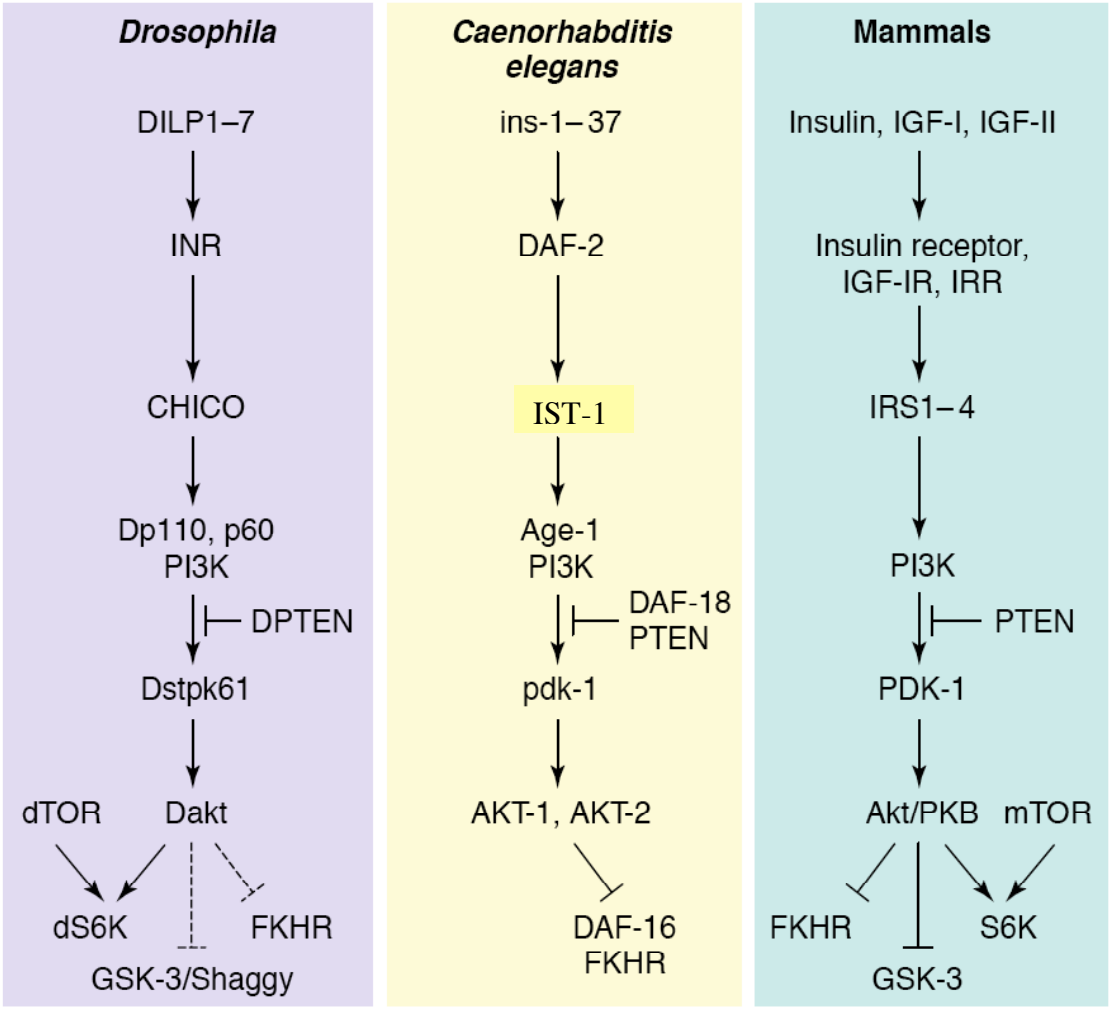
The first clue that insulin could be acting in the central nervous system to suppress feeding behavior came in 1979, when it was reported that intracerebroventricular (i.c.v.) infusion of insulin into baboons elicits both a decrease in food intake and body weight (Woods et al., 1979). These findings have since been validated in a number of other studies (Brief and Davis, 1984; Air et al., 2002). Conversely, neuron specific disruption of the insulin receptor gene in mice results in increased food intake and diet-sensitive obesity (Bruning et al., 2000). Insulin receptors are expressed throughout the central nervous system, including the hippocampus, a key brain region in the regulation of appetitive behaviors (Corp et al., 1986) as well as in the midbrain dopamine neurons of the ventral tegmental area (VTA) and the substantia nigra (Figlewicz et al., 2003). Interestingly, i.c.v. infusion of insulin in rats increases dopamine transporter mRNA levels in the VTA/substantia nigra (Figlewicz et al., 1994) and insulin administration can synergize with a subthreshold dose of the dopamine receptor antagonist raclopride to reduce the lick rates of a palatable sucrose solution (Baskin et al., 1999), suggesting that insulin may act on reward systems in the brain to decrease the rewarding aspects of food. Other possible downstream targets of insulin in the central nervous system include the hypothalamic peptides neuropeptide Y (Schwartz et al., 1992) and melanocortins (Benoit et al., 2002).

A number of studies have highlighted other neuronal roles for insulin. Besides supporting neuronal survival and differentiation (Robinson et al., 1994), insulin can also regulate neuropeptide secretion of egg-laying hormone from the bag cell neurons of *Aplysia* (Jonas et al., 1997). The IIS pathway has also been shown to play a part in axon guidance in *Drosophila*, where the insulin receptor is necessary for proper guidance of

photoreceptor cell axons from the retina to the brain during visual system development (Song et al., 2003). In addition, insulin administration can affect the translocation and recruitment of functional $GABA_A$ receptors to postsynaptic domains in hippocampal neuronal slices, producing a measurable change in $GABA_A$ receptor-mediated miniature inhibitory postsynaptic currents (mIPSCs) (Wan et al., 1997). Interestingly, in the field of alcohol research, overexpressing IGF-1 in the brain causes mice to become less sensitive to ethanol as measured in an ethanol-induced sleep assay, whereas inhibiting endogenous IGF-1 activity through overexpression of IGF binding protein 1 causes the opposite effect, increased ethanol-induced sleep (Pucilowski et al., 1996). Indeed, with roles ranging from regulation of food intake to axon guidance to neurotransmitter localization, the IIS pathway certainly keeps itself busy mediating a plethora of life processes.

Introductory Figure 1 (adapted from Garafolo, 2002)

The canonical insulin / insulin-like growth factor signaling (IIS) pathway



B.) The EGFR / ERK Signaling Pathway: Overview and Insights

Overview

Mitogen-activated protein (MAP) kinase signaling cascades have been shown to play essential roles in regulating a variety of cellular processes, including embryogenesis, cell differentiation and proliferation, cell death, and acute responses to hormones and environmental stresses (Chen et al., 2001; Pearson et al., 2001). Two of the major MAP kinase cascades, the c-Jun N-terminal kinase (JNK) pathway and the p38 pathway, play important roles in transducing cellular stress responses triggered by stimuli such as osmotic shock, heat shock, and inflammatory cytokines (Pearson et al., 2001). The other major and most well characterized MAP kinase pathway is the extracellular signal-regulated protein kinase (ERK) cascade, which is activated by growth factors, serum, and cytokines acting through receptors such as the epidermal growth factor (EGF) receptor (EGFR) (Pearson et al., 2001). The EGFR / ERK signaling pathway has been shown to regulate a variety of cellular processes, including the growth, differentiation, and proliferation of a variety of cell types (Yamada et al., 1997). In neurons, EGFR / ERK signaling has been shown to play a role in CNS neuron proliferation, migration, and differentiation, and plays a neurotrophic function in the survival of post-mitotic neurons (Xian and Zhou, 2004). In this section, I will describe the components of the EGFR / ERK signaling pathway and review how recent studies have revealed how neuronal EGFR / ERK pathway signaling has been found to regulate a variety of life processes,

including long-term potentiation, long-term memory, locomotor behaviors, and circadian rhythm. I will also describe *in vitro* and *in vivo* studies that have focused on the regulation of the EGFR / ERK pathway by ethanol.

To say that the EGFR / ERK signaling cascade has been well described would be an understatement (Kolch et al., 2002; Wong et al., 2004; Pearson et al., 2001; Yamada et al., 1997). For reference, please refer to the accompanying Introductory Figure 2 (from Kolch et al., 2002). Binding to the EGFR, a receptor tyrosine kinase, by ligands such as EGF or transforming growth factor-α (TGF-α), results in EGFR activation and autophosphorylation on tyrosine residues. Src homology 2 (SH2) domain containing molecules, including the adaptor proteins Grb2 and Shc, bind to these phosphorylated tyrosine residues with high affinity. Grb2, through its two src homology 3 (SH3) domains, then binds to the Ras guanine nucleotide exchange factor (GEF) Son of Sevenless (Sos), recruiting Sos to the plasma membrane where it can then induce the small membrane-localized G-protein Ras to exchange GDP for GTP, thus activating Ras. The activated G-protein Ras then recruits the MAPKKK Raf-1 from the cytosol to the membrane, where Raf-1 is activated through the phosphorylation of activating sites by various kinases such as PAK and Src and through the dephosphorylation of inhibitory sites by PP2A. Activated Raf-1 then phosphorylates and activates the MAPKK MEK, which in turn phosphorylates and activates the MAPK ERK. Activated ERK can then phosphorylate many cytosolic substrates, including protein kinases and phospholipase A_2. Phosphorylated ERK can also enter the nucleus, where it can modulate gene expression by phosphorylating various transcription factors including the Elk-1 and c-Myc.

It is noteworthy that the EGFR / ERK signaling cascade is well conserved between mammals and flies, and that most, if not all, of the canonical EGFR / ERK signaling cascade components described above have been identified in *Drosophila* (Perrimon and Perkins, 1997). In *Drosophila*, the EGFR / ERK pathway has been implicated in various phases of development, including the specification of cell fate in the central nervous system, germ band retraction in the embryo, and the development of the retina (Kumar et al., 1998; Perrimon and Perkins, 1997).

EGFR / ERK Pathway Regulation by Ethanol

In recent years, the intriguing relationship between ethanol and its regulation of EGFR / ERK pathway activity has been explored both *in vitro* and *in vivo*. In multiple different cell culture systems, including cultures of cortical neurons (Chandler and Sutton, 2005; Kalluri and Ticku, 2003), vascular smooth muscle cells (Hendrickson et al., 1998), hepatocytes (Sampey et al., 2007), and fibroblasts (Ma et al., 2005), acute exposure to ethanol results in strong inhibition of the phosphorylation of ERK in a dose dependent fashion, as measured by western blot analysis using an anti-phospho-ERK antibody. Similarly, measurement of activation levels of the EGFR using anti-phospho-EGFR antibodies show that ethanol inhibits EGF-induced EGFR phosphorylation as well (Ma et al., 2005). On the other hand, ethanol does not appear to affect the activation of two other MAP kinases, JNK and p38 (Ma et al., 2005). Interestingly, chronic treatment of cortical neuronal cultures with ethanol for 5 days has the opposite effect, that is, chronic ethanol treatment increases the phosphorylation levels of ERK (Kalluri and Ticku, 2003).

The inhibitory effect of acute ethanol treatment on ERK activation has been found to hold true *in vivo* as well, as acute intraperitoneal injection of various concentrations of ethanol ranging from 1.5 to 3.5 g/kg body weight in mice causes dose-dependent decreases in the phosphorylation of ERK in cortical neurons isolated from 5 to 60 minutes after ethanol injection (Kalluri and Ticku, 2002a). In a second study looking at ERK activation levels in various brain regions in differently aged rats, it was found that acute intraperitoneal injection of ethanol results in decreased levels of phospho-ERK in the cerebral cortex and hippocampus, but not the cerebellum, of postnatal day 5 and postnatal day 10 rats (Chandler and Sutton, 2005). In adult rats, however, ethanol injection results in a reduction of phospho-ERK in all three of these brain regions (Chandler and Sutton, 2005). In a third study, it was found that rats, after being subjected to continuous ethanol exposure for 12 days, have reduced ERK phosphorylation levels in various parts of the brain, including the frontal cortex, cerebellum, hippocampus, amygdala, and dorsal striatum (Sanna et al., 2002). Interestingly, when the animals are allowed to undergo withdrawal from ethanol exposure, ERK activation is significantly increased over baseline in these same brain regions (Sanna et al., 2002).

The mechanism through which ethanol might be inhibiting EGFR / ERK pathway activation is still very much under investigation, although several studies have suggested that ethanol may inhibit ERK phosphorylation by either activating $GABA_A$ receptors (Kalluri and Ticku, 2002a, 2002b) or by inhibiting NMDA receptors (Kalluri and Ticku, 2002a, 2003).

ERK Pathway Regulation of Long-term Potentiation and Long-term Memory

Several studies have revealed roles for ERK signaling in regulating both hippocampal- and amygdala- dependent long-term potentiation (LTP) and long-term memory. LTP, defined as a persistent increase in synaptic strength in response to brief pulses of high frequency electrical stimulation (Bliss and Lomo, 1973), has become widely used as a model for cellular learning and memory. English and Sweatt found that ERK phosphorylation levels in the CA1 region of the hippocampus are significantly increased as a result of LTP-inducing high frequency stimulation (English and Sweatt, 1996). To determine whether ERK activation is necessary for hippocampal LTP induction, the authors blocked ERK activation through the application of the pharamacological agent PD098059, an inhibitor of the MAPKK MEK (Dudley et al., 1995), and found that this markedly attenuates the induction of hippocampal LTP (English and Sweatt, 1997). Normal LTP induction in the amygdala has also been shown to be dependent on ERK pathway signaling, as inhibition of ERK pathway signaling using the MEK inhibitor U0126 blocks LTP in the amygdala (Huang and Kandel, 2007), and a similar perturbation in LTP induction in the amygdala is seen in knockout mice lacking the neuronal-specific guanine-nucleotide-exchange factor, Ras-GRF (Brambilla et al., 1997).

A regulatory role for ERK pathway signaling in hippocampal- and amygdala-dependent learning and memory has also been described. After training in a hippocampal dependent spatial memory task, the Morris water maze, ERK phosphorylation is increased in the pyramidal neurons of the dorsal hippocampus (Blum et al., 1999). Similarly, hippocampal ERK phosphorylation is significantly increased 1 hour after training in a contextual fear conditioning assay (Atkins et al., 1998). In an effort to

determine whether ERK activation is necessary for normal learning and memory, several groups have inhibited ERK phosphorylation through the application of pharmacological inhibitors and tested these animals behaviorally. Blocking ERK activation using the MEK inhibitor SL327 (Favata et al., 1998) blocks contextual fear conditioning in both rats and mice (Atkins et al., 1998; Selcher et al., 1999) and also impairs spatial learning in the Morris water maze (Selcher et al., 1999). While these studies suggest a role for ERK pathway activation in learning and the acquisition of memory, other studies contend that the ERK pathway is instead involved in the process of memory consolidation. For example, two groups that inhibited ERK signaling either by injecting rats with the MEK inhibitor PD098059 (Schafe et al., 1999) or by testing mice lacking Ras-GRF (Brambilla et al., 1997), found that these animals show normal memory after contextual and auditory conditioning when tested 30 minutes after conditioning but show impaired memory when tested 24 hours later (Schafe et al., 1999) Similarly, Blum et al. found that rats injected with PD098059 also show normal acquisition during training in the Morris water maze, but are impaired for memory retention when tested 48 hours later (Blum et al., 1999). While the exact role for ERK pathway signaling in regulating learning processes is clearly not yet resolved, what is apparent is that the ERK pathway does play significant roles in mediating induction of LTP and consolidation of long-term memory.

EGFR / ERK Pathway Regulation of Locomotor Patterns / Circadian Rhythm

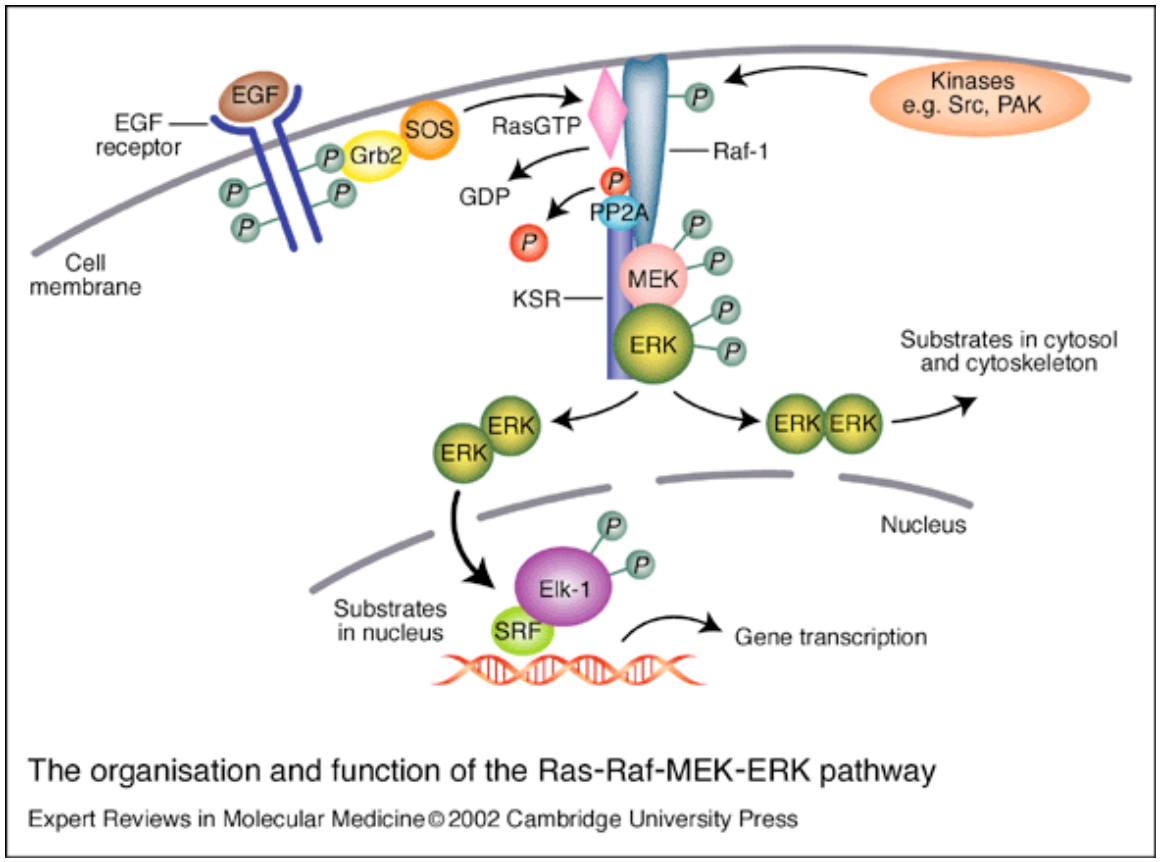
The first clue that the EGFR / ERK pathway might regulate sleep/waking behaviors in mammals came a decade ago, when it was found that intracerebroventricular injection of EGF into the brains of rabbits enhances time spent sleeping each day,

specifically enhancing non-rapid eye movement sleep (Kushikata et al., 1998). In mammals, the master circadian pacemaker is located in a region of the hypothalamus called the suprachiasmatic nucleus (SCN), a region that is believed to coordinate the circadian rhythms of behavior, such as the sleep-wake cycle, by secreting factors that act locally within the hypothalamus (Klein et al., 1991). Various studies over the years have found that both the EGFR and one of its ligands, transforming growth factor-α (TGF-α), are highly expressed in the SCN (Ma et al., 1994; Jobst et al., 2004; Van der Zee et al., 2005). Interestingly, one of these studies showed that levels of TGF-α immunoreactivity show circadian oscillations, peaking just prior to switches in light-dark conditions (Van der Zee et al., 2005). However, due to the high variability of the data, these trends were not statistically significant.

In a small-scale screen for secreted SCN factors that could affect locomotion in hamsters, it was found that infusion of the EGFR ligand TGF-α into the third ventricle of the hypothalamus results in a complete block of running-wheel activity during the three week-infusion, a phenotype that disappears upon cessation of the infusion (Kramer et al., 2001). In addition, hypothalamic infusion of TGF-α disrupts the timing of the circadian rhythm of sleep-wake behaviors in hamsters, manifested as an increase in the number of sleep-wake cycles per 24-hour period (Kramer et al., 2001), and also suppresses other active behaviors as well, such as grooming and feeding (Snodgrass-Belt et al., 2005). A similar effect on running-wheel activity was seen upon infusion of EGF, leading the authors to then examine the diurnal behavioral patterns of EGFR mutant mice. It was found that *waved-2* mutant mice, which bear a point mutation in the EGFR, causing an 80-95% decreases in ligand-stimulated receptor tyrosine kinase activity (Luetteke et al.,

1994), are abnormally active during the daytime, and show abnormally high locomotor responses to light pulses given during dark conditions (Kramer et al., 2001). A later study confirmed that EGFR activation in the SCN does indeed induce Erk1/2 phosphorylation in a rat ex vivo SCN slice culture system, supporting the hypothesis that TGF-α, secreted in a circadian fashion from the SCN, modulates circadian behaviors through activation of the EGFR / ERK pathway (Hao and Schwaber, 2006).

Introductory Figure 2 (from Kolch et al., 2002)



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Chapter 2

A Forward Genetic Screen for *Drosophila* Mutants with Altered Ethanol-Induced Locomotor Behavior

Ammon B. Corl and Aylin R. Rodan

Introduction

For decades the fruit fly, *Drosophila melanogaster*, has been the darling of geneticists around the world conducting forward genetic screens to identify novel molecules mediating various aspects of development and behavior. The joys of using *Drosophila* as a model organism are manifold (reviewed in St. Johnston, 2002). *Drosophila* are small, easy and cheap to rear, have a short 10-day generation time, and produce many, many progeny. In addition to being equipped with a model organism sporting a well-annotated genome bearing many genes with mammalian homologues, *Drosophila* geneticists have, at their disposal, a number of invaluable tools for the manipulation of gene expression both spatially and temporally through the GAL4-UAS binary expression system (Brand and Perrimon, 1993). The *Drosophila* genome can also be easily mutagenized for the purpose of large-scale genetic screens, whether by inducing point mutations through the feeding of ethyl methane sulphonate (EMS) or by the mobilization and random insertion of P transposable elements. P element-mediated mutagenesis has become increasingly popular due to the ease with which the mutated gene can be identified through techniques such as plasmid rescue or inverse PCR. Through the years, the cornucopia of wisdom garnered from such large-scale genetic screens has certainly been impressive, giving us a better understanding of the intricate pathways underlying various developmental processes such as embryo patterning and eye development (St. Johnston, 2002) as well as various behavioral processes such as courtship, learning and memory, circadian rhythm regulation, and drug-induced behaviors (reviewed in Sokolowki, 2001).

In an effort to identify and characterize novel genes and molecules involved in mediating drug-induced behaviors in *Drosophila*, several members of U. Heberlein's laboratory, over the course of approximately two years, created and screened a collection of approximately 2000 homozygous viable and 500 homozygous lethal P-element insertion lines in the *w* Berlin wild-type genetic background. Heberlein laboratory members screened for altered drug-induced behaviors in response to such drugs as nicotine and phencyclidine (a.k.a. PCP or angel dust). Ethanol, one of the most widely used and abused drugs in the world, was the drug of choice for several Heberlein laboratory members engaged in this forward genetic screen. Differing ethanol delivery paradigms were used to identify genes and molecules involved in mediating various ethanol induced behaviors, including loss of postural control (device used: inebriometer), hyperactivity (device used: booz-o-mat at a moderate ethanol concentration), and sedation (device used: booz-o-mat at a high ethanol concentration). In this chapter I will describe the results of one such P-element mutagenesis screen for genes and molecules involved in regulating ethanol induced behaviors at a relatively high concentration of ethanol, as measured in a high-throughput locomotor tracking device, the booz-o-mat.

Results

Overview of Screen and Criteria for Selection of Mutants and Controls

In an effort to identify genes and molecules involved in mediating the sedative effects of ethanol on behavior in *Drosophila melanogaster*, Aylin Rodan and Ammon Corl engaged in a forward genetic screen through the Heberlein laborabory's collection

of P[GAL4] insertions. Approximately 1500 P[GAL4] lines were screened in two discrete groups at a relatively high concentration of ethanol (100/50 E/A which corresponds to a relative flow of 100 U of ethanol vapor and 50 U of humidified air) using the booz-o-mat apparatus, an 8 chambered locomotor tracking device (Wolf et al., 2002). Group 1 consisted of 636 lines, which were initially screened by A. Rodan. After selecting and outcrossing 10 mutants and a set of 32 controls from these 636 lines, A. Rodan passed on these 42 lines to A. Corl for all further characterizations post-outcrossing, as reported in the subsection ***Group 1***, below. Group 2 consisted of 847 lines, which were initially screened by A. Corl. After selecting and outcrossing 11 mutants, A. Corl performed all further behavioral and molecular characterizations on the outcrossed mutants as described in subsection ***Group 2***, below.

As described previously (Wolf et al., 2002), when *Drosophila* are subjected to a continuous stream of ethanol vapors at a high ethanol concentration, they initially show a transient sharp increase in locomotor speed in response to the smell of ethanol termed the startle response. As the flies accumulate ethanol internally, their locomotor speed gradually slows and they eventually become akinetic and sedated. Analysis of a typical booz-o-mat "run" from the screen, where eight different genotypes are digitally videotaped simultaneously (see Methods), clearly shows this initial startle response and later sedative phase (Figure 1a). Occasionally, when analyzing a locomotor tracking profile, we would isolate a fly line whose tracking profile appeared different from the others in the same run (Figure 1b). This aberrant tracking profile was then compared to the mean tracking profile of the day. Lines that were judged to have a locomotor tracking phenotype differing substantially from the mean tracking profile, specifically differing by

at least 2 standard deviations from the mean at two or more consecutive time points, were selected for retesting (Figure 1c). Lines that showed consistently mutant tracking phenotypes over three or more days of testing were selected for outcrossing to both the *w* Berlin as well as the *w* Canton-S genetic backgrounds to eliminate unlinked modifiers. In addition, A. Rodan selected 32 control lines, which had exhibited tracking profiles similar to the mean tracking profile over multiple days, to both the *w* Berlin and the *w* Canton-S genetic backgrounds to generate a set of outcrossed control lines against which to compare the outcrossed mutant lines.

Group 1: 10 mutants

Note: Initial booz-o-mat screening of the 636 lines in Group 1 was performed by Aylin Rodan. All characterizations of Group 1 lines post-outcrossing, including booz-o-mat locomotor tracking, ethanol absorption assays, central nervous system (CNS) GAL4 expression analysis, and inverse PCR analysis were performed by Ammon Corl unless otherwise noted.

Group 1 mutant lines that retained their ethanol induced behavioral phenotypes post-outcrossing

Mutant 4-12a

Mutant 4-12a (II) was isolated due to its increased locomotor speed compared to controls at multiple time points in the booz-o-mat tracking profile, from 8 minutes to 20 minutes (Figure 2a). Post-outcrossing, 4-12a retained its phenotype in both the *w* Berlin

and the *w* Canton-S genetic backgrounds, displaying increased hyperactivity compared to controls for the entire second half of the tracking profile (Figure 2b, c, and d). Ethanol absorption assays demonstrated that 4-12a flies absorbed less ethanol compared to controls, suggesting that the observed ethanol induced behavioral phenotypes may be due, at least in part, to altered ethanol pharmacokinetics in 4-12a flies (Figure 2e). Analysis of the 4-12a CNS GAL4 expression pattern revealed expression primarily in the pars intercerebralis, median bundle, and suboesophageal ganglion regions of the brain. Inverse PCR analysis performed by Adrian Rothenfluh revealed the P-element of 4-12a to be inserted in the CG15626 gene region of chromosome 2L.

Mutant 7-65

Mutant 7-65 (I) was isolated due to its increased locomotor speed compared to controls throughout most of the booz-o-mat tracking profile, from 1.3 minutes to 20 minutes (Figure 3a). Post-outcrossing, 7-65 retained its phenotype in both the *w* Berlin and the *w* Canton-S genetic backgrounds, displaying increased hyperactivity throughout most of the tracking profile (Figure 3b, c, and d.) In the ethanol absorption assay, 7-65 flies tended to absorb less ethanol than the controls, although this difference was not statistically significant (Figure 3e). Analysis of the 7-65 CNS GAL4 expression pattern localized expression primarily in the mushroom bodies, ellipsoid body, and fan shaped body (Figure 3f). Inverse PCR analysis revealed the P-element in 7-65 to be inserted in the second exon of the gene *white rabbit (whir) / RhoGAP18B* at Flybase position 19047957 of the X chromosome. Mutant 7-65 and the *white rabbit* gene were further characterized by A. Rothenfluh (Rothenfluh et al., 2006).

Mutant 8-29

Mutant 8-29 (III) was isolated due to its increased locomotor speed in the middle of the tracking exposure, primarily from 6 - 15 minutes (Figure 4a.) In addition, 8-29 dispalyed a decreased startle response compared to controls (Figure 4a). Post-outcrossing, 8-29 retained its hyperactive phenotype in the *w* Berlin background (Figure 4b and d) but not in the *w* Canton-S background (Figure 4c). In addition, post-outcrossing to *w* Berlin, 8-29 displayed a relatively normal startle response when compared to controls 8-1, 8-12, and 8-165 (Figure 4d). 8-29 accumulated similar levels of ethanol compared to controls in the ethanol absorption assay, suggesting that the observed ethanol induced behavioral phenotypes were not due simply to altered ethanol pharmacokinetics (Figure 4e). Analysis of the 8-29 CNS GAL4 expression pattern revealed expression primarily localized to the mushroom bodies, antennal lobes, pars intercerebralis, and to a lesser extent the suboesophageal ganglion (Figure 4f). Inverse PCR analysis revealed the P-element in 8-29 to be inserted at Flybase position 860256 on chromosome 3R, approximately 45 kilobases (kb) upstream of *CG2022* and approximately 50 kb upstream of *corto*.

Mutant 10-184

Mutant 10-184 (I) was isolated due to its increased hyperactivity throughout its locomotor tracking profile (Figure 5a). Post-outcrossing, 10-184 retained its increased ethanol induced locomotor hyperactivity in both the *w* Berlin as well as the *w* Canton-S genetic backgrounds (Figure 5b, c, and d). Ethanol absorption assays revealed that 10-

184 absorbed less ethanol compared to controls, suggesting that the observed ethanol induced behavioral phenotypes may be due, at least in part, to altered ethanol pharmacokinetics in 10-184 flies (Figure 5e). The 10-184 CNS GAL4 expression pattern appeared to be highly concentrated in the pars intercerebralis region of the brain, with median bundle projections and other projections showing up faintly (Figure 5f). Inverse PCR analysis revealed the P-element in 10-184 to be inserted in the second exon of the gene *white rabbit (whir)* / *RhoGAP18B* at Flybase position 19047666 of the X chromosome. Mutant 10-184 and the *white rabbit* gene were further characterized by A. Rothenfluh (Rothenfluh et al., 2006).

Mutant 10-187

Mutant 10-187 (I) was isolated due to its increased hyperactivity commencing at about 3 minutes and enduring to the end of the ethanol exposure (Figure 6a). In addition, 10-187 displayed a decreased startle response compared to controls (Figure 6a). Post-outcrossing, 10-187 retained its mutant phenotypes in *w* Berlin (Figure 6b and d) but not in *w* Canton-S (Figure 6c). 10-187 accumulated similar levels of ethanol compared to controls in the ethanol absorption assay, suggesting that the observed ethanol induced behavioral phenotypes were not due simply to altered ethanol pharmacokinetics (Figure 6e). The 10-187 CNS GAL4 expression pattern appeared to be quite widespread, with especially strong expression in the optic lobes, mushroom bodies, antennal lobes, and pars intercerebralis (Figure 6f). Inverse PCR analysis revealed that the P-element in 10-187 is inserted at Flybase position 6761291 on the X chromosome, approximately 1.3 kb upstream of CG33691.

Group 1 mutant lines that did not retain their ethanol induced behavioral phenotypes post-outcrossing to *w* Berlin

Five of the ten mutants in Group 1 did not retain their ethanol induced behavioral phenotypes post-outcrossing to *w* Berlin. These mutants included: 3-11, 5-28, 8-47, 8-152, and 9-73. Mutant 3-11 (I) was isolated from the screen due to its weakly hyperactive locomotor response in the later half of the tracking profile (Figure 7a); after outcrossing to w Berlin it displayed tracking behavior similar to controls (Figure 7b). Mutant 5-28 (III) was isolated from the screen due to its weakly increased hyperactivity from 12.5 to 20 minutes in the locomotor tracking profile (Figure 7c); this hyperactivity was largely lost post-outcrossing (Figure 7d). Mutant 8-47 (III) was isolated from the screen due to its increased hyperactivity compared to controls on two of the five days in which it was tested (Figure 7e). After outcrossing to *w* Berlin 8-47 still displayed a tendency towards hyperactivity on two of the three days it was tested (Figure 7f). However, this hyperactivity was less than two standard deviations above the mean locomotor tracking response, and this mutant was deemed to have "lost" its behavioral phenotype post-outcrossing. Similarly, mutant 8-152 (III), which was isolated from the screen due to its increased hyperactivity on one of the three days on which it was tested (Figure 7g), also did not show increased hyperactivity compared to the mean + 2 standard deviations when tested post-outcrossing to *w* Berlin (Figure 7h). Finally, mutant 9-73 (I), which was isolated from the screen due to its increased hyperactivity from 3 to 15 minutes of ethanol

exposure (Figure 7i) completely lost its hyperactive phenotype post-outcrossing (Figure 7j).

Group 2: 11 mutants

Note: All characterizations of Group 2 mutants, including the initial screening through 847 P[GAL4] lines, were performed by Ammon Corl.

Group 2 mutant lines that retained their ethanol induced behavioral phenotypes post-outcrossing

Mutant 6-6

Mutant 6-6 (II) was initially isolated from the P[GAL4] screen due to its increased hyperactivity compared to controls during the second half of the locomotor tracking profile at a high concentration of ethanol (Figure 8a). Post-outcrossing, 6-6 retained its hyperactive phenotype in both the *w* Berlin (6-6B) as well as the *w* Canton-S (6-6CS) genetic backgrounds (Figure 8b and c). Interestingly, when tested for ethanol induced locomotor behavior at lower ethanol concentrations, 6-6B displayed a trend towards decreased hyperactivity, rather than increased hyperactivity, at earlier time points compared to controls (Figure 8d). 6-6B eluted out of the inebriometer at a rate comparable to that of controls (Figure 8e), and 6-6B flies showed normal geotaxis behavior compared to controls in a negative geotaxis assay (data not shown). When tested in an ethanol absorption assay, it was found that 6-6B absorbed less alcohol compared to controls at multiple time points, suggesting that the observed ethanol induced behavioral

phenotypes may be due, at least in part, to altered ethanol pharmacokinetics in 6-6B flies (Figure 8f). GAL4 expression analysis revealed a rather restricted 6-6B GAL4 expression pattern in the adult CNS, with expression confined primarily to the pars intercerebralis, median bundle, suboesophageal ganglion, and scattered cell clusters in the brain and ventral nerve cord (Figure 8g). Through inverse PCR it was found that the P-element in 6-6 is inserted at Flybase position 17267032 on chromosome IIR. The two nearest genes to the 6-6 insertion site are *CG10795* and *Acox57D-p*, located approximately 4 and 7 kb downstream of the 6-6 insertion site, respectively.

Mutant 8-222

Mutant 8-222 (II) was originally isolated from the P[GAL4] screen due to its hyperactivity during the second half of the locomotor tracking profile. This phenotype was somewhat variable; it was very strong during one day of testing and relatively weak on the other two days of testing (Figure 9a). Post-outcrossing to *w* Berlin, 8-222B showed reproducibly strong hyperactivity compared to controls on all three days of testing (Figure 9b). This hyperactive phenotype was not as pronounced in the *w* Canton-S genetic background (Figure 9c). When tested at various concentrations of ethanol, 8-222B behaved similarly to controls at both the 70/80 E/A and 120/30 E/A concentrations of ethanol, but showed a strong increase in hyperactivity over controls at the relatively low 50/100 concentration of ethanol (Figure 9d). In the inebriometer, 8-222B tended to elute later than the controls, although this difference was not statistically significant (Figure 9e). 8-222B behaved similarly to controls in the ethanol sedation assay (Figure 9f), the ethanol habituation assay (Figure 9g), as well as in the negative geotaxis assay

(data not shown). 8-222B accumulated similar levels of ethanol compared to controls in the ethanol absorption assay, suggesting that the observed ethanol induced behavioral phenotypes were not due simply to altered ethanol pharmacokinetics (Figure 9h). GAL4 expression analysis of 8-222B revealed CNS expression primarily in the mushroom bodies, the pars intercerebralis, the median bundle, fan shaped body, suboesophageal ganglion, and scattered neurons throughout the brain and ventral nerve cord (Figure 9i). The insertion location of the P-element inserted in 8-222, as determined by inverse PCR, is at Flybase position 131918 on chromosome II L, approximately 100 bp upstream of the first exon of *GS-1*, *Glutamine Synthetase 1*.

Mutant 9-34

Mutant 9-34 (II) was originally isolated from the P[GAL4] screen due to its weak hyperactivity during the first half of its locomotor tracking profile (Figure 10a). Post-outcrossing to *w* Berlin, the ethanol induced locomotor tracking phenotype of 9-34 Berlin (9-34B) became more refined, showing increased hyperactivity between 2.5 and 7.5 minutes and decreased hyperactivity compared to controls from 10 minutes onward (Figure 10b). In the *w* Canton-S background, 9-34CS appeared to have primarily lost its mutant locomotor tracking phenotype (Figure 10c). When tested at a relatively low concentration of ethanol, 50/100 E/A, 9-34B showed increased hyperactivity compared to controls at earlier time points in the tracking profile (Figure 10d). At a moderate concentration of ethanol, 70/80 E/A, 9-34B showed increased hyperactivity at the 3- and 4-minute time points in the tracking profile but showed decreased hyperactivity from 10 to 20 minutes (Figure 10d). 9-34B showed normal behavior when tested in the

inebriometer (Figure 10e), in the ethanol habituation assay (Figure 10f), and in the negative geotaxis assay (data not shown). 9-34B accumulated similar levels of ethanol compared to controls in the ethanol absorption assay, suggesting that the observed ethanol induced behavioral phenotypes were not due simply to altered ethanol pharmacokinetics (Figure 10g). 9-34B GAL4 expression in the adult CNS was restricted to just a few bilaterally symmetric cell clusters in the brain, including cells in the pars intercerebralis region (Figure 10h). Inverse PCR analysis revealed the 9-34 P-element to be inserted at Flybase position 2628194 on chromosome II R, located approximately 200 bp upstream of *l(2)01289*, a gene predicted by Flybase to encode a protein with protein disulfide isomerase activity, as well as approximately 1 kb upstream of *phtf*, a putative homeodomain transcriptional factor.

Mutant 17-3

Mutant 17-3 (III) was isolated from the P[GAL4] screen due to its strong hyperactivity at various time points during its 100/50 E/A ethanol induced locomotor tracking profile (Figure 11a). This hyperactive phenotype persisted post-outcrossing, although the phenotype was more pronounced in the *w* Berlin genetic background (17-3B, Figure 11b) compared to the *w* Canton-S genetic background (17-3CS, Figure 11c). 17-3B showed an increased hyperactivity compared to controls at various concentrations of ethanol tested (Figure 11d). Unlike any of the other mutants in Group 2, 17-3B flies showed an increased baseline locomotor activity compared to controls in the absence of ethanol vapors (Figure 11d, bottom panel), suggesting that 17-3 flies may simply be generally hyperactive and that the hyperactivity phenotypes seen during screening may

not be ethanol specific. 17-3B flies eluted out of the inebriometer with a mean elution time that was not statistically different from those of controls (Figure 11e) and performed normally in a negative geotaxis assay (data not shown). 17-3B flies accumulated similar levels of ethanol compared to controls in the ethanol absorption assay, suggesting that the observed ethanol induced behavioral phenotypes were not due simply to altered ethanol pharmacokinetics (Figure 11f). 17-3B CNS GAL4 expression appeared to be pan-neuronal, with GAL4 expression strongest in the pars intercerebralis, mushroom bodies, ellipsoid body, fan shaped body, antennal lobes, and suboesophageal ganglion (Figure 11g). Inverse PCR analysis revealed the 17-3 P-element to be inserted at Flybase position 4883047 on chromosome III R, in the second intron of *CG11033*.

Mutant 17-51

Mutant 17-51 (II) was isolated from the P[GAL4] screen due to its increased hyperactivity during the second half of its locomotor tracking profile at 100/50 E/A, a relatively high and sedating concentration of ethanol (Figure 12a). Post-outcrossing, 17-51 retained this hyperactivity in the *w* Berlin genetic background (17-51B, Figure 12b) but not in the *w* Canton-S genetic background (17-51CS, Figure 12c). At a relatively low concentration of ethanol, 50/100 E/A, 17-51B flies showed increased hyperactivity at three and four minutes into the ethanol exposure, but looked similar to controls at all other time points (Figure 12d, top panel). 17-51B flies looked similar to controls at all time points at moderate concentration of ethanol, 70/80 E/A (Figure 12d, middle panel). At a high, sedating concentration of ethanol, 120/30 E/A, 17-51B flies again showed increased hyperactivity compared to controls during the second half of the locomotor

tracking profile (Figure 12d, bottom panel), leading to the hypothesis that 17-51 flies may be resistant to ethanol induced sedation. Indeed, when tested in the sedation assay, 17-51B flies showed strong resistance to sedation compared to controls at a number of ethanol concentrations tested (Figure 12e and data not shown). In addition, 17-51B flies showed faster habituation when tested in the ethanol habituation assay (Figure 12f). 17-51B flies eluted out of the inebriometer at a rate similar to that of controls (Figure 12g) and performed normally in the negative geotaxis assay (data not shown). 17-51B flies accumulated similar levels of ethanol compared to controls in the ethanol absorption assay, suggesting that the observed ethanol induced behavioral phenotypes were not due simply to altered ethanol pharmacokinetics (Figure 12h). 17-51B CNS GAL4 expression appeared to be pan-neuronal, with GAL4 expression strongest in the pars intercerebralis, mushroom bodies, ellipsoid body, antennal lobes, and suboesophageal ganglion (Figure 12i). Inverse PCR analysis revealed the 17-51 P-element to be inserted at Flybase position 15066249 on chromosome II R, 10 base pairs upstream of the first exon of gene *CG7097*. Further characterization of mutant 17-51 and gene *CG7097* can be found in Chapter 3 of this thesis.

Group 2 mutant lines that did not retain their ethanol induced behavioral phenotypes post-outcrossing to *w* Berlin

Six of the eleven mutants in Group 2 did not retain their ethanol induced behavioral phenotypes post-outcrossing to *w* Berlin. These mutants included: 5-41, 9-43, 10-251, 10-256, 10-282, and 11-100x. Mutant 5-41x was originally isolated from the P[GAL4] screen due to its increased hyperactivity from 4 to 10 minutes during the booz-

o-mat locomotor tracking assay (Figure 13a); this hyperactive phenotype was lost post-outcrossing (Figure 13b). Mutant 9-43 was isolated from the screen due to its increased hyperactivity from 7.5 to 12.5 minutes of tracking (Figure 13c). Post-outcrossing to *w* Berlin, 9-43 still retained a weakly hyperactive phenotype (Figure 13d); however, this phenotype was no longer greater than two standard deviations above the mean tracking profile. Mutant 10-251, post-outcrossing to *w* Berlin, completely lost the hyperactive tracking phenotype it had displayed prior to being outcrossed (compare Figure 13e to Figure 13f). Mutant 10-256 was isolated from the P[GAL4] screen due to a conspicuous spike in hyperactivity at the 1.3 and 2.5 minute time points of ethanol exposure (Figure 13g). This spike in hyperactivity was completely lost post-outcrossing to *w* Berlin, although the outcrossed 10-256 did show a modest increase in hyperactivity at later points in the tracking profile (Figure 13h). Mutant 10-282 was isolated from the screen due to its increased hyperactivity during the first half of its tracking profile (Figure 13i); this mutant phenotype was not observed post-outcrossing to *w* Berlin (Figure 13j). Finally, mutant 11-100x was isolated from the P[GAL4] screen due to its increased hyperactivity during the later half of the tracking profile (Figure 13k). Post-outcrossing, rather than showing increased hyperactivity, 11-100x tended to show modestly decreased hyperactivity during the second half of the locomotor tracking profile (Figure 13l).

Discussion

A forward genetic screen for *Drosophila* mutants displaying altered ethanol induced locomotor behaviors was conducted using a high-throughput locomotor tracking device, the booz-o-mat. A relatively high, sedating concentration of ethanol was chosen

for this screen (100/50 E/A) in an effort to preferentially isolate mutants resistant to the sedative effects of ethanol. Of the approximately 1500 P[GAL4] lines screened using this assay, 21 mutants were selected for their hyperactive locomotor tracking profiles and were outcrossed to *w* Berlin and *w* Canton-S in order to eliminate unlinked modifiers. The percent of mutant lines isolated from this particular screen (approximately 1.4% of all lines screened) is comparable to those obtained from previous behavioral screens aimed at isolating *Drosophila* mutants with altered drug-induced behaviors (Tsai et al., 2004; Scholtz et al., 2005). About half of these lines (11 out of 21) did not retain their ethanol-induced locomotor tracking phenotypes post-outcrossing to *w* Berlin, suggesting that the locomotor tracking phenotypes observed in the un-outcrossed strains were due to modifiers unlinked to the P element insertion, e.g. those induced by genomic "scarring" caused by multiple insertions and remobilizations of the GawB element. 10 of these 21 mutants retained their aberrant behavioral phenotypes post-outcrossing. These lines were then subjected to various other behavioral tests post-outcrossing, including locomotor tracking in the booz-o-mat at varying concentrations of ethanol, testing for loss of postural control in the inebriometer, measuring the development of sedation in the sedation assay, and habituation development in the ethanol vapor pulse habituation assay. Inverse PCR revealed the genomic insertion points of the P-elements inserted in these 10 mutants, giving insight as to which nearby genes might be responsible for mediating the observed ethanol induced mutant phenotypes. Some of these P[GAL4] mutants contain P-elements in or near known and studied genes, such as Glutamine Synthetase 1 (mutant 8-222) and RhoGAP18B (mutants 7-65 and 10-184). In other cases, the P-elements are inserted near genes of unknown function or are inserted relatively far away from any

identified gene locus. Ethanol absorption assays revealed that in some of these mutants, specifically mutants 4-12a, 10-184, and 6-6, the observed behavioral phenotypes may be due, in part at least, to altered ethanol pharmacokinetics. Meanwhile, driving and visualizing UAS-GFP expression with the GAL4 activity of the inserted P[GAL4] elements allowed us to hypothesize which neuronal subsets might normally express the genes being affected by these P-element mutations.

While several tantalizing mutants were isolated as a result of this P[GAL4] screen for fly lines displaying altered ethanol induced behaviors at a high concentration of ethanol, ultimately I had to select only one mutant for further characterization. After much deliberating, I chose to study mutant 17-51, a fly line that showed a strong resistance to ethanol induced sedation at various ethanol vapor concentrations. The characterization of mutant 17-51 is described in detail in Chapter 3 of this thesis. Hopefully, the other mutants isolated from this screen will be studied in more detail as well, lending further insight into the molecular mechanisms that mediate ethanol induced behaviors in *Drosophila*.

Materials and Methods

Drosophila culture and strains

Genetic Screen and Selection of Controls Approximately 1500 P[GAL4] insertions (carrying the GawB element, Brand et al., 1994) in the *w* Berlin genetic background were screened as homozygotes in the booz-o-mat at a 100/50 E/A concentration as described below. Two members of Ulrike Heberlein's lab carried out

this screen: 636 lines were screened by Aylin Rodan, a former graduate student, and 847 lines were screened by Ammon Corl. After each day of screening, each fly line's EtOH induced locomotor tracking profile was compared to the mean tracking profile of the day. Lines that were judged to have a locomotor tracking phenotype differing substantially from the mean tracking profile, specifically differing by at least 2 standard deviations from the mean at two or more consecutive timepoints, were selected for retesting. Lines that showed consistently mutant tracking phenotypes over three or more days of testing were selected for outcrossing.

From A. Rodan's screen of 636 P[GAL4] lines, 10 lines were chosen for outcrossing to both the *w* Berlin as well as the *w* Canton-S genetic backgrounds. These lines were: 4-12a, 7-65, 8-29, 10-184, 5-28, 8-47, 3-11, 9-73, 10-187, and 8-152. In addition to outcrossing these ten lines, A. Rodan also selected and outcrossed a set of 32 control lines, which had exhibited tracking profiles similar to the mean tracking profile over multiple days, to both the *w* Berlin and the *w* Canton-S genetic backgrounds in order to generate a set of outcrossed control lines against which to compare the outcrossed mutant lines. These 32 control lines included: 3-26, 3-69, 3-85, 3-89, 4-26, 4-48, 4-58, 4-74, 4-85, 7-27, 7-42, 7-51, 7-82, 8-1, 8-12, 8-51, 8-79, 8-87, 8-89, 8-142, 8-165, 8-188, 8-200, 10-6, 10-8, 10-67, 10-69, 10-84, 10-89, 10-191, 10-197, and 10-198. After outcrossing these 10 mutant lines and 32 control lines, A. Rodan passed on all 42 outcrossed stocks to A. Corl for all further characterizations.

From A. Corl's screen of ~850 P[GAL4] lines, 11 lines were chosen for outcrossing to both the *w* Berlin as well as the *w* Canton-S genetic backgrounds. These

lines were: 10-256, 9-43, 5-41x, 10-251, 8-222, 10-282, 11-100x, 9-34, 6-6, 17-3, and 17-51.

Post-outcrossing, mutant lines were compared initially to the mean tracking profiles of all 32 controls outcrossed by A. Rodan. In later experiments, mutant lines were compared to just three of the 32 controls: 8-1, 8-12, and 8-165.

For locomotor tracking experiments at a moderate concentration of alcohol (70/80 E/A), mutants were compared to three P[GAL4] controls, outcrossed 5 times to the *w* Berlin background, graciously provided by Fred Wolf. These controls were: 3-54, 3-89, and 4-81 and were originally generated as part of the Heberlein lab P[GAL4] screen collection.

For inebriometer experiments, mutants were compared to three P[GAL4] controls, outcrossed 5 times to the *w* Berlin background, graciously provided by Douglas Guarnieri. These controls were: 3-54, 5-120, and 10-149 and were generated as part of the Heberlein lab P[GAL4] screen collection.

For habituation experiments, mutants were compared to two P[GAL4] controls, outcrossed 5 times to the *w* Berlin background, graciously provided by Mark Eddison. These controls were: 4-59 and 16-57 and were generated as part of the Heberlein lab P[GAL4] screen collection.

Lines for the Heberlein lab P[GAL4] screen collection were generated through the combined efforts of Melissa Andres, Françoise Chanut, William Cho, Ammon Corl, Mark Eddison, Douglas Guarnieri, Josh Niclas, Aylin Rodan, Adrian Rothenfluh, Fred Wolf, and Katherine Woo.

Behavioral Assays

Flies were raised on standard cornmeal and molasses food at 25°C and 70% relative humidity. All experiments were performed on 2-5 day old males at 20°C, utilizing 25 males for each behavioral run. All genotypes were tested across multiple days. All lines tested in behavioral experiments were in the same genetic background, *w* Berlin, with the exception of certain locomotor tracking assays performed post-outcrossing, where the genetic background was *w* Canton-S.

Locomotor Tracking Assay Locomotor tracking assays were performed in the "booz-o-mat" as described previously (Wolf et al., 2002). Briefly, twenty-five 2-4 day old males of each genotype were introduced to the chambers in the booz-o-mat. Flies were allowed to equilibrate in humidified air for 10 minutes before digital camera filming commenced. The motion of the flies was then recorded for 2 minutes in humidified air, followed by 21 minutes in an ethanol/air mixture of 50/100 E/A, 70/80 E/A, 100/50 E/A, 110/40 E/A, or 120/30 E/A. Films were then analyzed with a modified version of DIAS 3.2 (Solltech, Oakdale, IA) and the average speed of the flies was plotted as a function of time.

Ethanol Sedation Assay Twenty-five 2-4 day old males of each genotype were introduced to the chambers in the booz-o-mat. After being given 12 minutes of humidified air to equilibrate to the apparatus, the flies were given a continuous stream of ethanol vapors (110/40 E/A) for 30 minutes. During this thirty period time period, flies were visually assayed for sedation at 11 time points. At each time point, the flies were

given a mechanical stimulus (each tube containing flies was twirled within each booz-o-mat chamber) and the number of flies that were lying immobile at the bottom of each tube post twirling were scored as being sedated. The assayer was blinded to the identity of the genotypes for the course of the sedation assay.

Inebriometer Testing Flies were tested in the inebriometer as described previously (Moore et al., 1998). Inebriometers were set to an ethanol/humidified air mixture of ~60/35 E/A and were allowed to equilibrate to 20°C. Flies were allowed to equilibrate 5 minutes at 20°C before being introduced into the inebriometer. As flies eluted from the inebriometer, they were counted in 3-minute bins by a *Drosophila* activity monitor (Trikinetics, Waltham, MA). Mean elution times (METs) were then calculated from the resulting elution profiles for the various genotypes tested.

Habituation Assay Flies were tested in a habituation assay as described previously (Cho et al., 2004). Briefly, twenty male flies of each genotype were introduced into a modified, four chambered booz-o-mat locomotor tracking apparatus. After being allowed to acclimate in humidified air for 7 minutes, video camera filming commenced, and flies were exposed to a series of thirty-second pulses of a 70/80 E/A mixture. Between pulses, flies were exposed to a flow of humidified air. Following the fourth pulse of ethanol vapors, flies were given a sudden dishabituating mechanical stimulus, followed three minutes later by one more ethanol pulse. Films were then analyzed with a modified version of DIAS 3.2 (Solltech, Oakdale, IA) and the average speed of the flies was plotted as a function of ethanol pulse number.

Negative Geotaxis Assay The negative geotaxis assay, which also measures locomotion and responsiveness to banging, was performed as described previously (Moore et al., 1998) with the following exceptions. The dimensions for the cylinder used were 22.5 cm in length by 2.7 cm in width. After loading the ten 2-4 day old flies of each genotype into a cylinder, the flies were banged down to the bottom and observed as they climbed to the top. At the end of each one-minute period, the number of flies that had reached the top of the cylinder was counted and the flies were banged down once more. This process was repeated at 1-minute intervals for 5 minutes.

Ethanol Absorption Assay

Groups of twenty-five flies of each genotype were exposed to an ethanol / humidified air mixture of 100/50 E/A for 0, 5, 10, 15, or 20 minutes in perforated test tubes in the booz-o-mat apparatus (Wolf et al., 2002). Following exposure to ethanol, flies were frozen on dry ice and homogenized in 500 μL of 50 mM Tris-HCl (pH 7.5). Ethanol assays were then performed on the fly homogenates as previously described (Moore et al., 1998).

Histology

Light Microscopy. To visualize GAL4 expression patterns, P[GAL4] virgins were crossed to males of a line carrying the GFP reporter gene under the control of a UAS enhancer element (cytoplasmic GFP: UAS-GFP T2 – Bloomington; microtubule associated GFP: UAS-GFP-tau – Y.-N. Jan). After dissecting out the brain and ventral

nerve cord of 2-4 day old adult male progeny in 1xPBS, specimens were washed in 1xPBS and visualized using a Zeiss Axioskop II microscope.

Molecular Biology

The genomic DNA flanking the P[GAL4] insertions was isolated using inverse PCR. Comparisons with the genome sequence of *Drosophila* on Flybase (www.flybase.org) revealed the genomic locations of the various P[GAL4] insertions.

Statistics

Statistical significance was established using either Student's *t*-tests assuming equal variance or one-way analysis of variance (ANOVA) tests followed by post-hoc Newman-Keuls testing using GraphPad Prism software, Version 4 (Graphpad, San Diego, CA). Error bars in all experiments represent the standard error of the mean (s.e.m.).

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Figure Legends

Figure 1: Sample booz-o-mat tracking profiles from the P[GAL4] screen at a relatively high (100/50 E/A) concentration of ethanol.

1a.) A sample booz-o-mat "run" showing the locomotor tracking profiles of 8 different genotypes during a 20 minute continuous exposure of ethanol vapors. Notice that early on in the tracking profiles the various genotypes exhibit a locomotor "startle" response then gradually decrease their speed until they become immobile and sedated.

1b.) A second sample booz-o-mat run showing the behavior of mutant line 11.100x compared to 6 other genotypes. Note that 11.100x was more hyperactive compared to the other 6 lines tested at various time points in the locomotor tracking profile.

1c.) The locomotor tracking profile of 11.100x is plotted against the mean locomotor tracking profile of all flies tested on 12/12/01 plus and minus two standard deviations. Note that 11.100x is more hyperactive than the mean tracking profile plus two standard deviations, flagging this line as one to retest / outcross.

Figure 2: Characterization of Mutant 4-12a

2a.) The ethanol-induced locomotor tracking profiles of mutant 4-12a, pre-outcrossing, on three days of testing are plotted alongside the average tracking profile of all other P[GAL4] lines tested on those days. The concentration of ethanol used was 100/50 E/A.

2b.) The ethanol-induced locomotor tracking profiles of mutant 4-12aB, post-outcrossing to the *w* Berlin genetic background, on three days of testing are plotted alongside the average tracking profile of a set of 32 P[GAL4] control lines tested on those days. The concentration of ethanol used was 100/50 E/A.

2c.) The ethanol-induced locomotor tracking profiles of mutant 4-12aCS, post-outcrossing to the *w* Canton-S genetic background, on three days of testing are plotted alongside the average tracking profile of a set of 32 P[GAL4] control lines tested on those days. The concentration of ethanol used was 100/50 E/A.

2d.) The average ethanol-induced locomotor tracking profile of mutant 4-12aB, post-outcrossing to the *w* Berlin genetic background, is plotted alongside the average tracking profiles of three P[GAL4] control lines: 8-1B, 8-12B, and 8-165B. The concentration of ethanol used was 100/50 E/A. n=3. In this and all subsequent figures, error bars represent standard error of the mean (s.e.m.).

2e.) After being exposed to 21 minutes of ethanol vapors in the booz-o-mat apparatus (100/50 E/A), 4-12aB flies show decreased internal concentrations of ethanol compared to control lines. One-way ANOVA revealed a significant difference between genotypes ($p=0.0113$). Post-hoc Newman-Keuls analysis revealed a difference between 4-12aB and 8-165B ($p<0.01$), between 4-12aB and 8-1B ($p<0.05$), and between 4-12aB and 8-12B ($p<0.05$). n=9.

2f.) The 4-12a CNS GAL4 expression pattern as visualized by GFP-T2 fluorescence. GAL4 expression can be observed in the pars intercerebralis (PI), median bundle (Med. B.), and suboesophageal ganglion (SEG).

Figure 3: Characterization of Mutant 7-65

3a.) The ethanol-induced locomotor tracking profiles of mutant 7-65, pre-outcrossing, on three days of testing are plotted alongside the average tracking profile of all other P[GAL4] lines tested on those days. The concentration of ethanol used was 100/50 E/A.

3b.) The ethanol-induced locomotor tracking profiles of mutant 7-65B, post-outcrossing to the *w* Berlin genetic background, on three days of testing are plotted alongside the average tracking profile of a set of 32 P[GAL4] control lines tested on those days. The concentration of ethanol used was 100/50 E/A.

3c.) The ethanol-induced locomotor tracking profiles of mutant 7-65CS, post-outcrossing to the *w* Canton-S genetic background, on three days of testing are plotted alongside the average tracking profile of a set of 32 P[GAL4] control lines tested on those days. The concentration of ethanol used was 100/50 E/A.

3d.) The average ethanol-induced locomotor tracking profile of mutant 7-65B, post-outcrossing to the *w* Berlin genetic background, is plotted alongside the average tracking profiles of three P[GAL4] control lines: 8-1B, 8-12B, and 8-165B. n=3. The concentration of ethanol used was 100/50 E/A.

3e.) After being exposed to 21 minutes of ethanol vapors in the booz-o-mat apparatus (100/50 E/A), 7-65B flies have internal ethanol concentrations that are not statistically different from those of controls. One-way ANOVA analysis failed to reveal a difference between genotypes ($p=0.0505$). n=9.

3f.) The 7-65 CNS GAL4 expression pattern as visualized by GFP-T2 fluorescence. GAL4 expression can be observed in the fan shaped body (FSB), the mushroom bodies (MB), and the ellipsoid body (EB).

Figure 4: Characterization of Mutant 8-29

4a.) The ethanol-induced locomotor tracking profiles of mutant 8-29, pre-outcrossing, on five days of testing are plotted alongside the average tracking profile of all other P[GAL4] lines tested on those days. The concentration of ethanol used was 100/50 E/A.

4b.) The ethanol-induced locomotor tracking profiles of mutant 8-29B, post-outcrossing to the *w* Berlin genetic background, on three days of testing are plotted alongside the average tracking profile of a set of 32 P[GAL4] control lines tested on those days. The concentration of ethanol used was 100/50 E/A.

4c.) The ethanol-induced locomotor tracking profiles of mutant 8-29CS, post-outcrossing to the *w* Canton-S genetic background, on three days of testing are plotted alongside the

average tracking profile of a set of 32 P[GAL4] control lines tested on those days. The concentration of ethanol used was 100/50 E/A.

4d.) The average ethanol-induced locomotor tracking profile of mutant 8-29B, post-outcrossing to the *w* Berlin genetic background, is plotted alongside the average tracking profiles of three P[GAL4] control lines: 8-1B, 8-12B, and 8-165B. The concentration of ethanol used was 100/50 E/A. n=3.

4e.) After being exposed to 21 minutes of ethanol vapors in the booz-o-mat apparatus (100/50 E/A), 8-29B flies have internal ethanol concentrations that are not statistically different from those of controls. One-way ANOVA analysis failed to reveal a difference between genotypes ($p=0.3267$). n=9.

4f.) The 8-29 CNS GAL4 expression pattern as visualized by GFP-T2 fluorescence. GAL4 expression can be observed in the pars intercerebralis (PI), the mushroom bodies (MB), the antennal lobes (AL), and the suboesophageal ganglion (SEG).

Figure 5: Characterization of Mutant 10-184

5a.) The ethanol-induced locomotor tracking profiles of mutant 10-184, pre-outcrossing, on three days of testing are plotted alongside the average tracking profile of all other P[GAL4] lines tested on those days. The concentration of ethanol used was 100/50 E/A.

5b.) The ethanol-induced locomotor tracking profiles of mutant 10-184B, post-outcrossing to the *w* Berlin genetic background, on three days of testing are plotted alongside the average tracking profile of a set of 32 P[GAL4] control lines tested on those days. The concentration of ethanol used was 100/50 E/A.

5c.) The ethanol-induced locomotor tracking profiles of mutant 10-184CS, post-outcrossing to the *w* Canton-S genetic background, on three days of testing are plotted alongside the average tracking profile of a set of 32 P[GAL4] control lines tested on those days. The concentration of ethanol used was 100/50 E/A.

5d.) The average ethanol-induced locomotor tracking profile of mutant 10-184B, post-outcrossing to the *w* Berlin genetic background, is plotted alongside the average tracking profiles of three P[GAL4] control lines: 8-1B, 8-12B, and 8-165B. The concentration of ethanol used was 100/50 E/A. n=3.

5e.) After being exposed to 21 minutes of ethanol vapors in the booz-o-mat apparatus (100/50 E/A), 10-184B flies show decreased internal concentrations of ethanol compared to control lines. One-way ANOVA revealed a significant difference between genotypes ($p=0.0022$). Post-hoc Newman-Keuls analysis revealed a difference between 10-184B and 8-165B ($p<0.01$), between 10-184B and 8-1B ($p<0.01$), and between 10-184B and 8-12B ($p<0.01$). n=9.

5f.) The 10-184 CNS GAL4 expression pattern as visualized by GFP-T2 fluorescence (left panel) and GFP-tau fluorescence (right panel). GAL4 expression can be observed in the pars intercerebralis (PI), and median bundle (Med. B.).

Figure 6: Characterization of Mutant 10-187

6a.) The ethanol-induced locomotor tracking profiles of mutant 10-187, pre-outcrossing, on three days of testing are plotted alongside the average tracking profile of all other P[GAL4] lines tested on those days. The concentration of ethanol used was 100/50 E/A.

6b.) The ethanol-induced locomotor tracking profiles of mutant 10-187B, post-outcrossing to the *w* Berlin genetic background, on three days of testing are plotted alongside the average tracking profile of a set of 32 P[GAL4] control lines tested on those days. The concentration of ethanol used was 100/50 E/A.

6c.) The ethanol-induced locomotor tracking profiles of mutant 10-187CS, post-outcrossing to the *w* Canton-S genetic background, on three days of testing are plotted alongside the average tracking profile of a set of 32 P[GAL4] control lines tested on those days. The concentration of ethanol used was 100/50 E/A.

6d.) The average ethanol-induced locomotor tracking profile of mutant 10-187B, post-outcrossing to the *w* Berlin genetic background, is plotted alongside the average tracking profiles of three P[GAL4] control lines: 8-1B, 8-12B, and 8-165B. The concentration of ethanol used was 100/50 E/A. n=3.

6e.) After being exposed to 21 minutes of ethanol vapors in the booz-o-mat apparatus (100/50 E/A), 10-187B flies have internal ethanol concentrations that are not statistically different from those of controls. One-way ANOVA analysis failed to reveal a difference between genotypes ($p=0.7898$). n=9.

6f.) The 10-187 CNS GAL4 expression pattern as visualized by GFP-T2 fluorescence. GAL4 expression can be observed in the pars intercerebralis (PI), the mushroom bodies (MB), the antennal lobes (AL), and the optic lobes (OL).

Figure 7: Group 1 lines that did not retain their ethanol-induced locomotor tracking phenotypes post-outcrossing to *w* Berlin.

Figures 7a, 7c, 7e, 7g, and 7i.): The ethanol-induced locomotor tracking profiles of each mutant line, pre-outcrossing, on two to five days of testing are plotted alongside the average tracking profile of all other P[GAL4] lines tested on those days. The concentration of ethanol used was 100/50 E/A.

Figures 7b, 7d, 7f, 7h, and 7j.) The ethanol-induced locomotor tracking profiles of each mutant line, post-outcrossing to the *w* Berlin genetic background, on three days of testing are plotted alongside the average tracking profile of a set of 32 P[GAL4] control lines tested on those days. The concentration of ethanol used was 100/50 E/A.

7a.) and 7b.): The ethanol-induced locomotor tracking phenotypes of mutant 3-11 compared to controls both pre-outcrossing (Figure 7a.) and post-outcrossing to *w* Berlin (Figure 7b.).

7c.) and 7d.): The ethanol-induced locomotor tracking phenotypes of mutant 5-28 compared to controls both pre-outcrossing (Figure 7c.) and post-outcrossing to *w* Berlin (Figure 7d.).

7e.) and 7f.): The ethanol-induced locomotor tracking phenotypes of mutant 8-47 compared to controls both pre-outcrossing (Figure 7e.) and post-outcrossing to *w* Berlin (Figure 7f.).

7g.) and 7h.): The ethanol-induced locomotor tracking phenotypes of mutant 8-152 compared to controls both pre-outcrossing (Figure 7g.) and post-outcrossing to *w* Berlin (Figure 7h.).

7i.) and 7j.): The ethanol-induced locomotor tracking phenotypes of mutant 9-73 compared to controls both pre-outcrossing (Figure 7i.) and post-outcrossing to *w* Berlin (Figure 7j.).

Figure 8: Characterization of Mutant 6-6

8a.) The ethanol-induced locomotor tracking profiles of mutant 6-6, pre-outcrossing, on three days of testing are plotted alongside the average tracking profile of all other P[GAL4] lines tested on those days. The concentration of ethanol used was 100/50 E/A.

8b.) The ethanol-induced locomotor tracking profiles of mutant 6-6B, post-outcrossing to the *w* Berlin genetic background, on three days of testing are plotted alongside the average tracking profile of a set of 32 P[GAL4] control lines tested on those days. The concentration of ethanol used was 100/50 E/A.

8c.) The ethanol-induced locomotor tracking profiles of mutant 6-6CS, post-outcrossing to the *w* Canton-S genetic background, on three days of testing are plotted alongside the average tracking profile of a set of 32 P[GAL4] control lines tested on those days. The concentration of ethanol used was 100/50 E/A.

8d.) The average ethanol-induced locomotor tracking profile of mutant 6-6B, post-outcrossing to the *w* Berlin genetic background, is plotted alongside the average tracking profiles of P[GAL4] control lines. Flies were tracked at a low concentration of ethanol (50/100 E/A, top panel) and at a moderate concentration of ethanol (70/80 E/A, bottom panel). n=5-6.

8e.) 6-6B mutant flies eluted from the inebriometer with a mean elution time (MET) comparable to those of controls. One-way ANOVA analysis failed to reveal a significant difference between genotypes ($p=0.0879$). n=4.

8f.) 6-6B mutant flies have alterations in ethanol pharmacokinetics compared to controls. Ethanol levels were measured in extracts of 6-6B flies and control flies (see Methods). Ethanol exposure was continuous starting at time = 0. One-way ANOVAs followed by post-hoc Newman-Keuls analysis revealed significant differences between 6-6B and controls at the 5 minute ($p=0.0181$), 10 minute ($p=0.0104$), and 15 minute (0.0039) time points. n=4.

8g.) The 6-6 CNS GAL4 expression pattern as visualized by GFP-T2 fluorescence. GAL4 expression can be observed in the pars intercerebralis (PI), the median bundle (Med. B.), and the suboesophageal ganglion (SEG).

Figure 9: Characterization of Mutant 8-222

9a.) The ethanol-induced locomotor tracking profiles of mutant 8-222, pre-outcrossing, on three days of testing are plotted alongside the average tracking profile of all other P[GAL4] lines tested on those days. The concentration of ethanol used was 100/50 E/A.

9b.) The ethanol-induced locomotor tracking profiles of mutant 8-222B, post-outcrossing to the *w* Berlin genetic background, on three days of testing are plotted alongside the average tracking profile of a set of 32 P[GAL4] control lines tested on those days. The concentration of ethanol used was 100/50 E/A.

9c.) The ethanol-induced locomotor tracking profiles of mutant 8-222CS, post-outcrossing to the *w* Canton-S genetic background, on three days of testing are plotted alongside the average tracking profile of a set of 32 P[GAL4] control lines tested on those days. The concentration of ethanol used was 100/50 E/A.

9d.) The average ethanol-induced locomotor tracking profile of mutant 8-222B, post-outcrossing to the *w* Berlin genetic background, is plotted alongside the average tracking profiles of P[GAL4] control lines. Flies were tracked at a low concentration of ethanol (50/100 E/A, top panel), at a moderate concentration of ethanol (70/80 E/A, middle panel), and at a high concentration of ethanol (120/30 E/A, bottom panel). n=5-6.

9e.) 8-222B mutant flies eluted from the inebriometer with a mean elution time (MET) slightly higher than those of controls. One-way ANOVA analysis revealed a significant difference between genotypes ($p=0.0254$). Post-hoc Newman-Keuls analysis revealed a significant difference between 6-6 and one of the controls, 5-120 ($p<0.05$), but did not reveal significant differences between 6-6 and the other two controls, 3-54 ($p>0.05$) and 10-149 ($p>0.05$). n=4.

9f.) 8-222B flies, when tested in the booz-o-mat sedation assay at a high concentration of ethanol (110/40 E/A), sedate at a rate comparable to those of controls. n=8.

9g.) 8-222B flies, when tested in the ethanol vapor pulse habituation assay, habituate similarly to controls. n=8.

9h.) 8-222B flies do not have alterations in ethanol pharmacokinetics. Ethanol levels were measured in extracts of flies of 8-222B and control lines 8-1B, 8-12B, and 8-165B (see Methods). Ethanol exposure was continuous starting at time = 0. n=4.

9i.) The 8-222 CNS GAL4 expression pattern as visualized by GFP-T2 fluorescence. GAL4 expression can be observed in the pars intercerebralis (PI), the median bundle (Med. B.), the suboesophageal ganglion (SEG), the fan shaped body (FSB), and the mushroom bodies (MB).

Figure 10: Characterization of Mutant 9-34

10a.) The ethanol-induced locomotor tracking profiles of mutant 9-34, pre-outcrossing, on three days of testing are plotted alongside the average tracking profile of all other P[GAL4] lines tested on those days. The concentration of ethanol used was 100/50 E/A.

10b.) The ethanol-induced locomotor tracking profiles of mutant 9-34B, post-outcrossing to the *w* Berlin genetic background, on three days of testing are plotted alongside the average tracking profile of a set of 32 P[GAL4] control lines tested on those days. The concentration of ethanol used was 100/50 E/A.

10c.) The ethanol-induced locomotor tracking profiles of mutant 9-34CS, post-outcrossing to the *w* Canton-S genetic background, on three days of testing are plotted

alongside the average tracking profile of a set of 32 P[GAL4] control lines tested on those days. The concentration of ethanol used was 100/50 E/A.

10d.) The average ethanol-induced locomotor tracking profile of mutant 9-34B, post-outcrossing to the *w* Berlin genetic background, is plotted alongside the average tracking profiles of P[GAL4] control lines. Flies were tracked at a low concentration of ethanol (50/100 E/A, top panel), at a moderate concentration of ethanol (70/80 E/A, middle panel), and at a high concentration of ethanol (120/30 E/A, bottom panel). n=5-6.

10e.) 9-34B mutant flies eluted from the inebriometer with a mean elution time (MET) comparable to those of controls. One-way ANOVA analysis failed to reveal a significant difference between genotypes ($p=0.1098$). n=4.

10f.) 9-34B flies, when tested in the ethanol vapor pulse habituation assay, habituate similarly to controls. n=8.

10g.) 9-34B flies do not have alterations in ethanol pharmacokinetics. Ethanol levels were measured in extracts of flies of 9-34B and control lines 8-1B, 8-12B, and 8-165B (see Methods). Ethanol exposure was continuous starting at time = 0. n=4.

10h.) The 9-34 CNS GAL4 expression pattern as visualized by GFP-T2 fluorescence. GAL4 expression can be observed in just a few pairs of bilaterally symmetric cells, including cells in the pars intercerebralis (PI).

Figure 11: Characterization of Mutant 17-3

11a.) The ethanol-induced locomotor tracking profiles of mutant 17-3, pre-outcrossing, on three days of testing are plotted alongside the average tracking profile of all other P[GAL4] lines tested on those days. The concentration of ethanol used was 100/50 E/A.

11b.) The ethanol-induced locomotor tracking profiles of mutant 17-3B, post-outcrossing to the *w* Berlin genetic background, on three days of testing are plotted alongside the average tracking profile of a set of 32 P[GAL4] control lines tested on those days. The concentration of ethanol used was 100/50 E/A.

11c.) The ethanol-induced locomotor tracking profiles of mutant 17-3CS, post-outcrossing to the *w* Canton-S genetic background, on three days of testing are plotted alongside the average tracking profile of a set of 32 P[GAL4] control lines tested on those days. The concentration of ethanol used was 100/50 E/A.

11d.) The average ethanol-induced locomotor tracking profile of mutant 17-3B, post-outcrossing to the *w* Berlin genetic background, is plotted alongside the average tracking profiles of P[GAL4] control lines. Flies were tracked at a low concentration of ethanol (50/100 E/A, top panel), at a moderate concentration of ethanol (70/80 E/A, middle panel), and at a high concentration of ethanol (120/30 E/A, bottom panel). n=5-6.

11e.) 17-3B mutant flies eluted from the inebriometer with a mean elution time (MET) comparable to those of controls. One-way ANOVA analysis failed to reveal a significant difference between genotypes ($p=0.0707$). n=4.

11f.) 17-3B flies do not have alterations in ethanol pharmacokinetics. Ethanol levels were measured in extracts of flies of 9-34B and control lines 8-1B, 8-12B, and 8-165B (see Methods). Ethanol exposure was continuous starting at time = 0. n=4.

11g.) The 17-3 CNS GAL4 expression pattern as visualized by GFP-T2 fluorescence. GAL4 expression appears to be strongest in the pars intercerebralis (PI), the mushroom bodies (MB), the antennal lobes (AL), the ellipsoid body (EB), and the suboesophageal ganglion (SEG).

Figure 12: Characterization of Mutant 17-51

12a.) The ethanol-induced locomotor tracking profiles of mutant 17-51, pre-outcrossing, on three days of testing are plotted alongside the average tracking profile of all other P[GAL4] lines tested on those days. The concentration of ethanol used was 100/50 E/A.

12b.) The ethanol-induced locomotor tracking profiles of mutant 17-51B, post-outcrossing to the *w* Berlin genetic background, on three days of testing are plotted alongside the average tracking profile of a set of 32 P[GAL4] control lines tested on those days. The concentration of ethanol used was 100/50 E/A.

12c.) The ethanol-induced locomotor tracking profiles of mutant 17-51CS, post-outcrossing to the *w* Canton-S genetic background, on three days of testing are plotted alongside the average tracking profile of a set of 32 P[GAL4] control lines tested on those days. The concentration of ethanol used was 100/50 E/A.

12d.) The average ethanol-induced locomotor tracking profile of mutant 17-51B, post-outcrossing to the *w* Berlin genetic background, is plotted alongside the average tracking profiles of P[GAL4] control lines. Flies were tracked at a low concentration of ethanol (50/100 E/A, top panel), at a moderate concentration of ethanol (70/80 E/A, middle panel), and at a high concentration of ethanol (120/30 E/A, bottom panel). n=5-6.

12e.) 17-51B flies, when tested in the booz-o-mat sedation assay at a high concentration of ethanol (110/40 E/A) were resistant to ethanol induced sedation compared to controls from 12.5 minutes to 30 minutes of exposure. n=8.

12f.) 17-51B flies, when tested in the ethanol vapor pulse habituation assay, habituated more rapidly than controls. One-way ANOVA across ethanol vapor pulse 2 revealed a significant difference between genotypes ($p=0.0019$). Post-hoc Newman-Keuls analysis revealed a significant difference between 17-51B and 4-59B ($p<0.01$) and between 17-51B and 16-57B ($p<0.05$). n=12.

12g.) 17-51B mutant flies eluted from the inebriometer with a mean elution time (MET) comparable to those of controls. One-way ANOVA analysis failed to reveal a significant difference between genotypes ($p=0.0728$). n=4.

12h.) 17-51B flies do not have alterations in ethanol pharmacokinetics. Ethanol levels were measured in extracts of flies of 17-51B and control lines 8-1B, 8-12B, and 8-165B (see Methods). Ethanol exposure was continuous starting at time = 0. n=4.

12i.) The 17-51 CNS GAL4 expression pattern as visualized by GFP-T2 fluorescence. GAL4 expression appears to be strongest in the pars intercerebralis (PI), the mushroom bodies (MB), the antennal lobes (AL), the ellipsoid body (EB), and the suboesophageal ganglion (SEG).

Figure 13: Group 2 lines that did not retain their ethanol-induced locomotor tracking phenotypes post-outcrossing to *w* Berlin.

Figures 13a, 13c, 13e, 13g, 13i, and 13k.): The ethanol-induced locomotor tracking profiles of each mutant line, pre-outcrossing, on three days of testing are plotted alongside the average tracking profile of all other P[GAL4] lines tested on those days. The concentration of ethanol used was 100/50 E/A.

Figures 13b, 13d, 13f, 13h, 13j, and 13l.) The ethanol-induced locomotor tracking profiles of each mutant line, post-outcrossing to the *w* Berlin genetic background, on three days of testing are plotted alongside the average tracking profile of a set of 32

P[GAL4] control lines tested on those days. The concentration of ethanol used was 100/50 E/A.

13a.) and 13b.): The ethanol-induced locomotor tracking phenotypes of mutant 5-41x compared to controls both pre-outcrossing (Figure 13a.) and post-outcrossing to *w* Berlin (Figure 13b.).

13c.) and 13d.): The ethanol-induced locomotor tracking phenotypes of mutant 9-43 compared to controls both pre-outcrossing (Figure 13c.) and post-outcrossing to *w* Berlin (Figure 13d.).

13e.) and 13f.): The ethanol-induced locomotor tracking phenotypes of mutant 10-251 compared to controls both pre-outcrossing (Figure 13e.) and post-outcrossing to *w* Berlin (Figure 13f.).

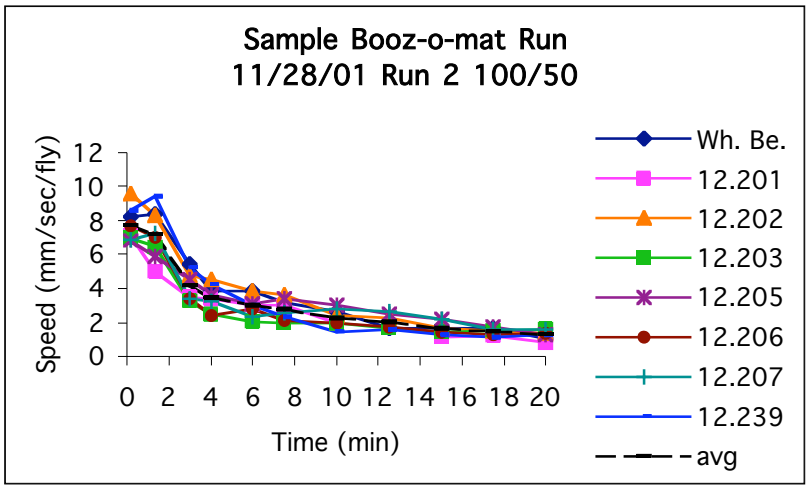
13g.) and 13h.): The ethanol-induced locomotor tracking phenotypes of mutant 10-256 compared to controls both pre-outcrossing (Figure 13g.) and post-outcrossing to *w* Berlin (Figure 13h.).

13i.) and 13j.): The ethanol-induced locomotor tracking phenotypes of mutant 10-282 compared to controls both pre-outcrossing (Figure 13i.) and post-outcrossing to *w* Berlin (Figure 13j.).

13k.) and 13l.): The ethanol-induced locomotor tracking phenotypes of mutant 11-100x compared to controls both pre-outcrossing (Figure 13k.) and post-outcrossing to *w* Berlin (Figure 13l.).

Figure 1

A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11/28/01 Run 2 100/50
Speed (mm/sec/fly)
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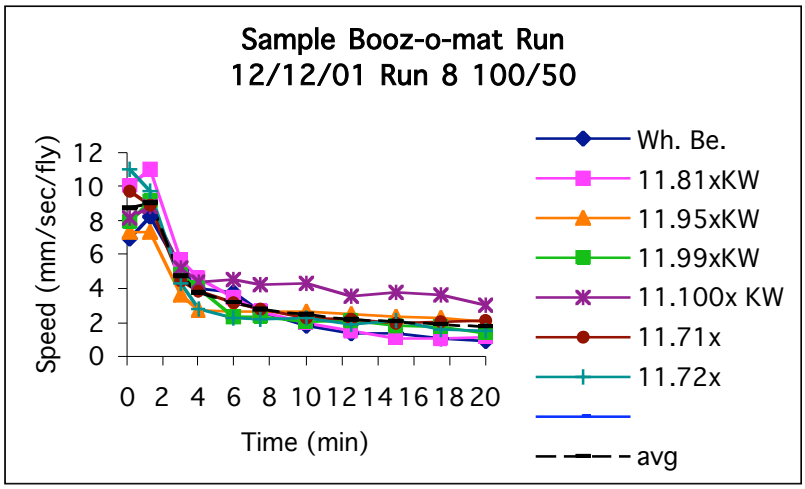
0 2 4 6 8 10 12 14 16 18 20
Time (min)
Wh. Be.
12.201
12.202
12.203
12.205
12.206
12.207
12.239
avg

B


Sample Booz-o-mat Run
12/12/01 Run 8 100/50
Speed (mm/sec/fly)
0 2 4 6 8 10 12
0 2 4 6 8 10 12 14 16 18 20
Time (min)
Wh. Be.
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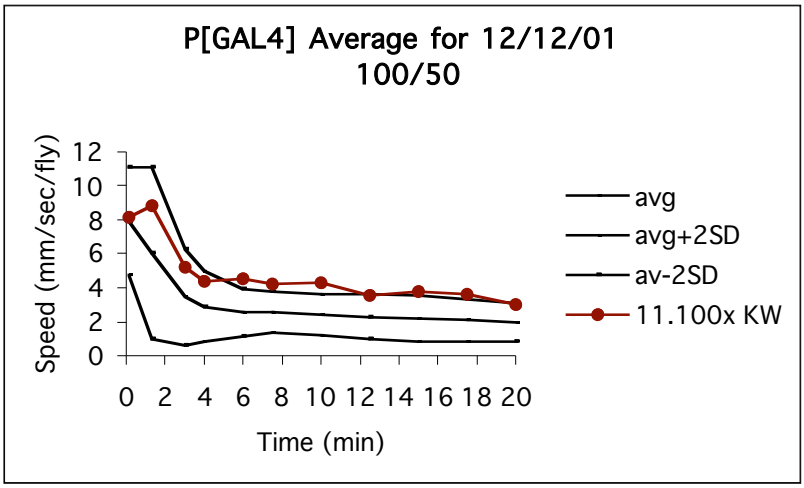
11.95xKW
11.99xKW
11.100x KW
11.71x
11.72x
avg

C


P[GAL4] Average for 12/12/01
100/50
Speed (mm/sec/fly)
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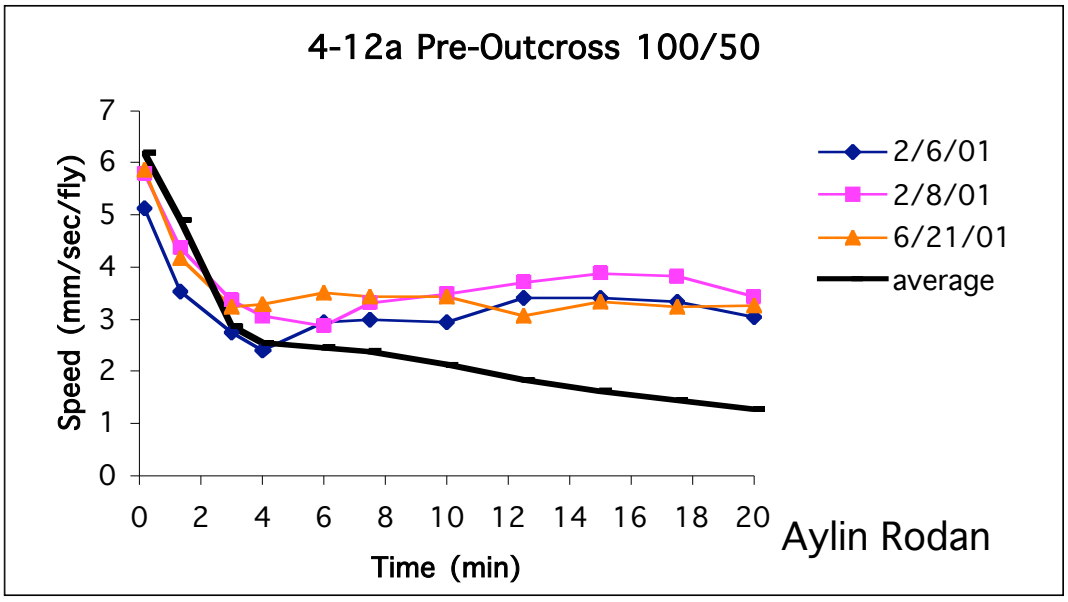
0 2 4 6 8 10 12 14 16 18 20
Time (min)
avg
avg+2SD
av-2SD
11.100x KW

Figure 2

A


4-12a Pre-Outcross 100/50
Speed (mm/sec/fly)
Time (min)
2/6/01
2/8/01
6/21/01
average
Aylin Rodan

B

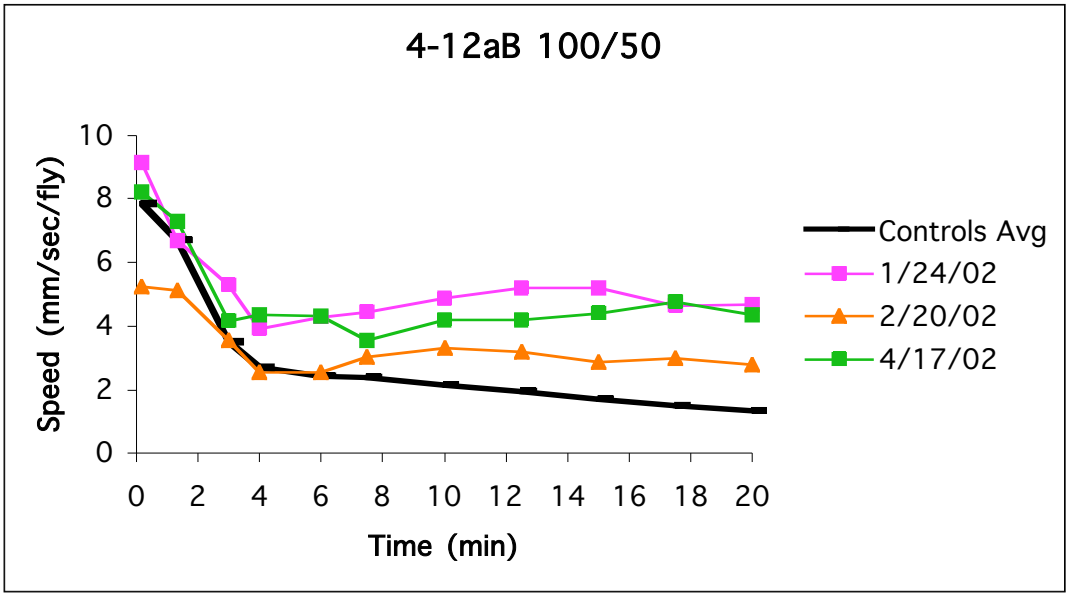
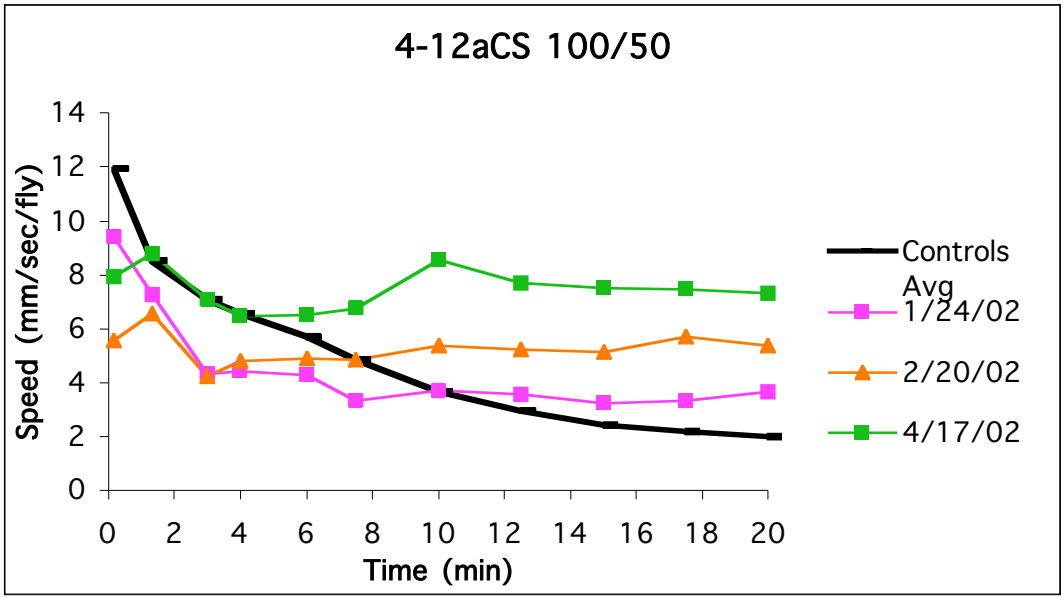

4-12aB 100/50
Speed (mm/sec/fly)
Time (min)
Controls Avg
1/24/02
2/20/02
4/17/02

Figure 2

C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Speed (mm/sec/fly)
Time (min)
Controls Avg
1/24/02
2/20/02
4/17/02

D

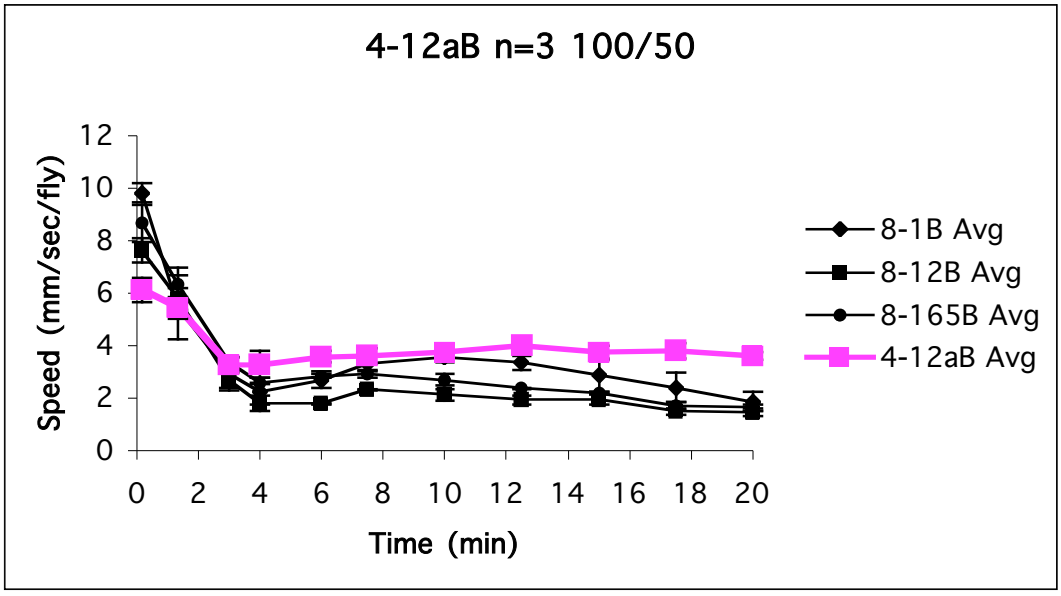
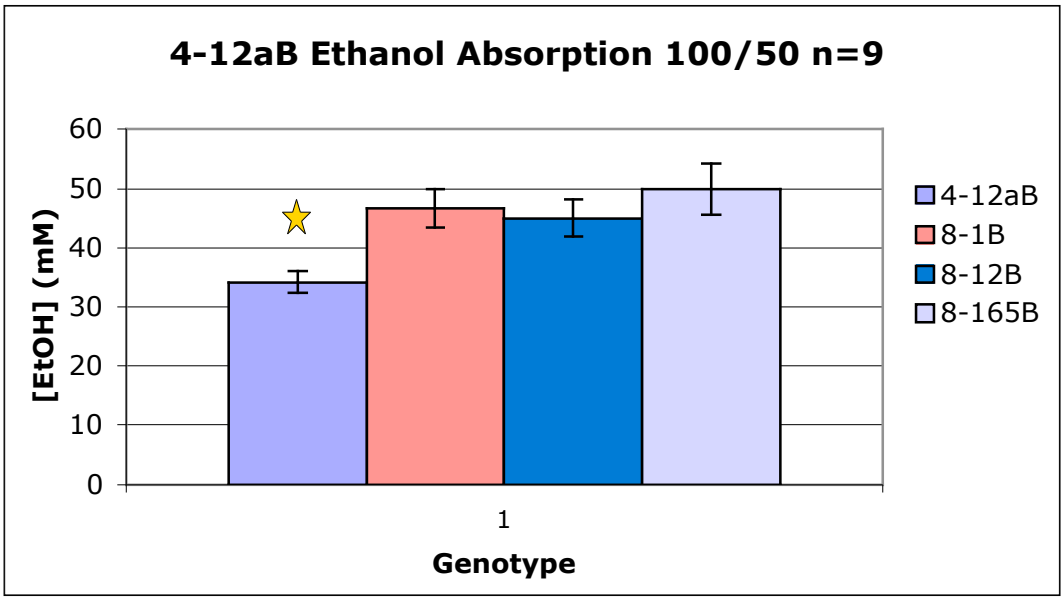
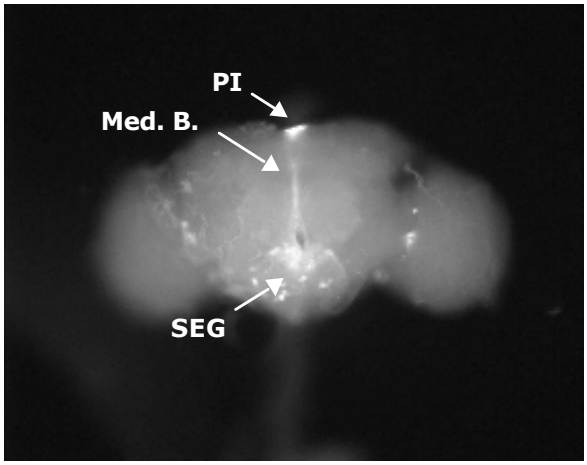

4-12aB n=3 100/50
Speed (mm/sec/fly)
Time (min)
8-1B Avg
8-12B Avg
8-165B Avg
4-12aB Avg

Figure 2

E


4-12aB Ethanol Absorption 100/50 n=9
[EtOH] (mM)
60
50
40
30
20
10
0
1
Genotype
4-12aB
8-1B
8-12B
8-165B

F


PI
Med. B.
SEG

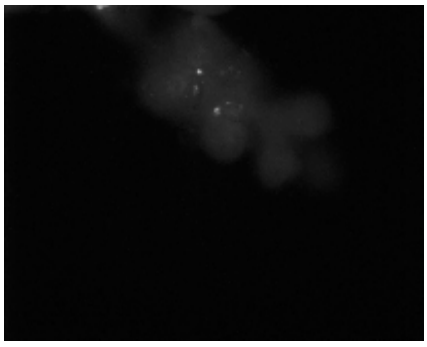
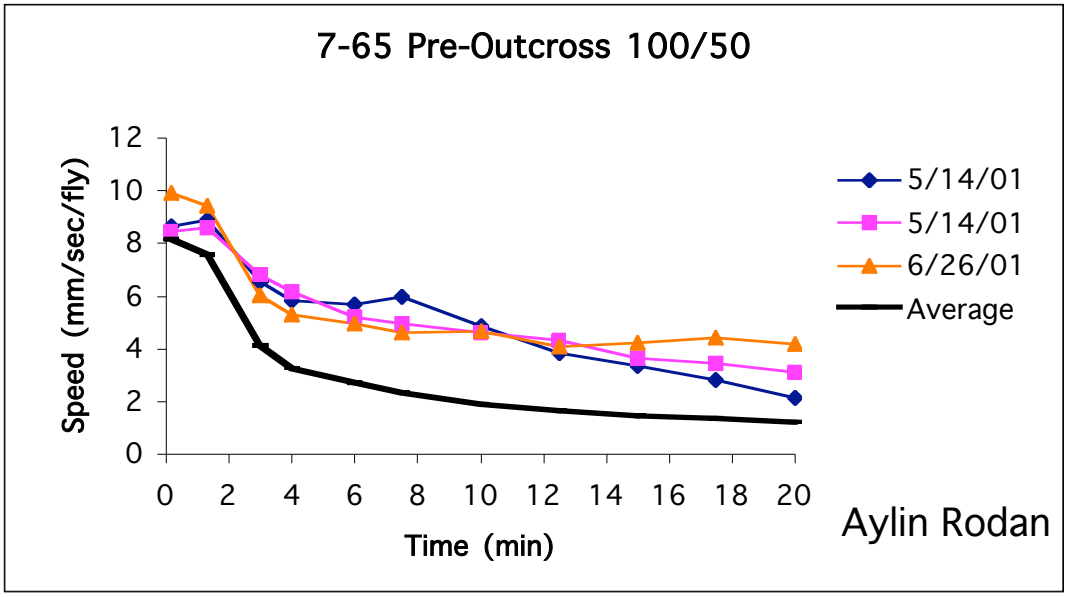


Figure 3

A


7-65 Pre-Outcross 100/50
Speed (mm/sec/fly)
Time (min)
5/14/01
5/14/01
6/26/01
Average
Aylin Rodan

B

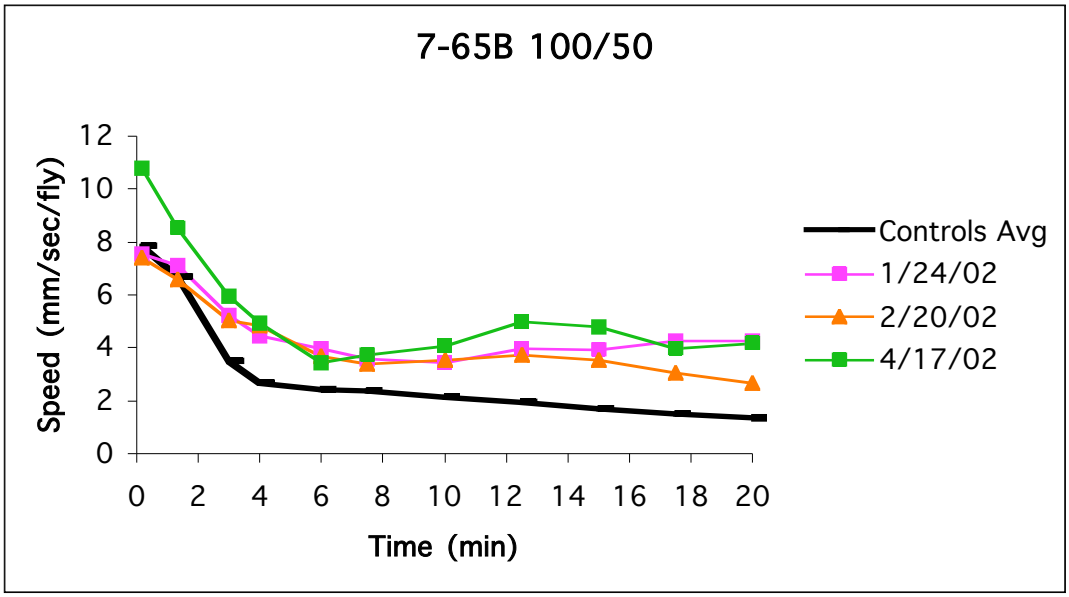
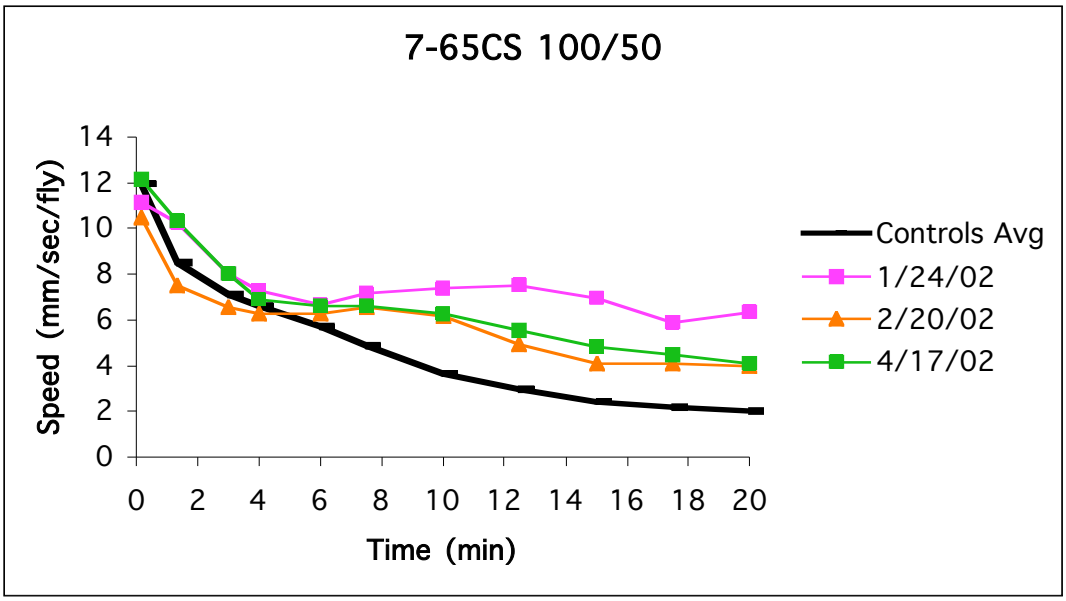

7-65B 100/50
Speed (mm/sec/fly)
Time (min)
Controls Avg
1/24/02
2/20/02
4/17/02

Figure 3

C


7-65CS 100/50
Speed (mm/sec/fly)
Time (min)
Controls Avg
1/24/02
2/20/02
4/17/02

D

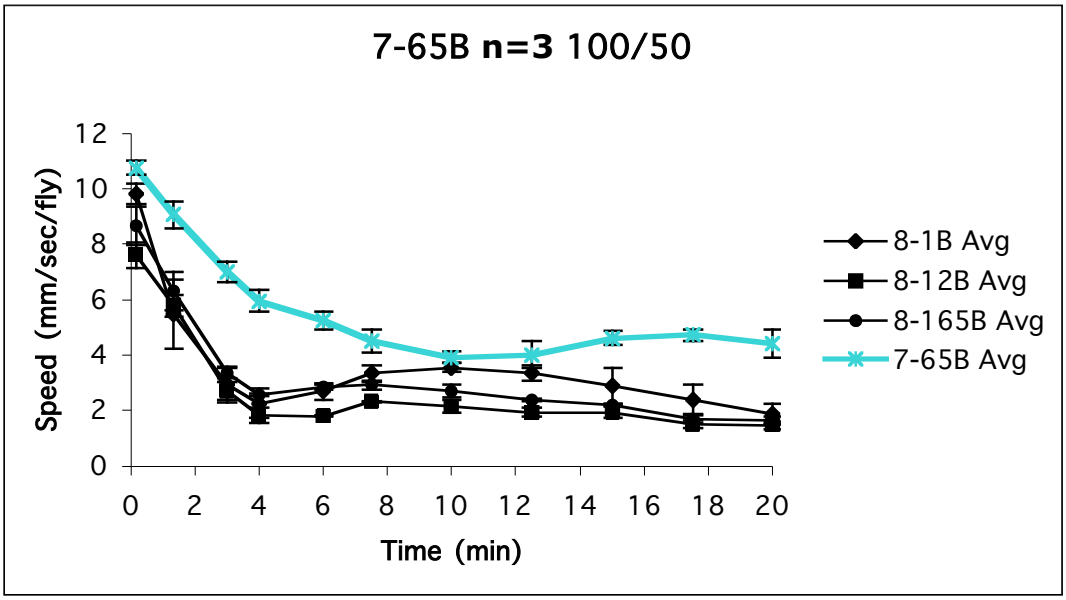
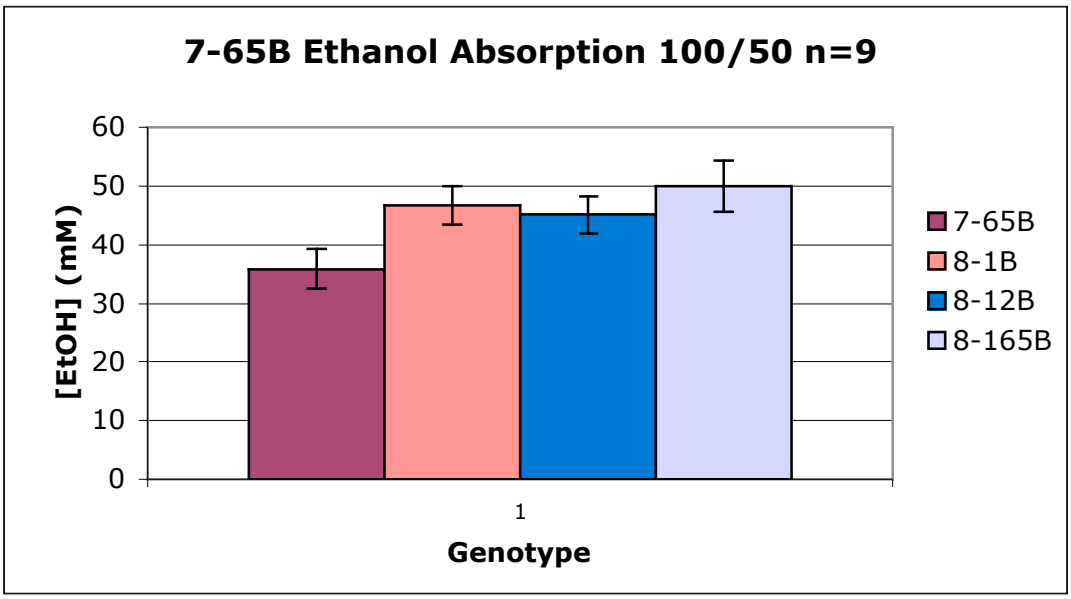

7-65B n=3 100/50
Speed (mm/sec/fly)
Time (min)
8-1B Avg
8-12B Avg
8-165B Avg
7-65B Avg

Figure 3

E


7-65B Ethanol Absorption 100/50 n=9
[EtOH] (mM)
60
50
40
30
20
10
0
1
Genotype
7-65B
8-1B
8-12B
8-165B

F

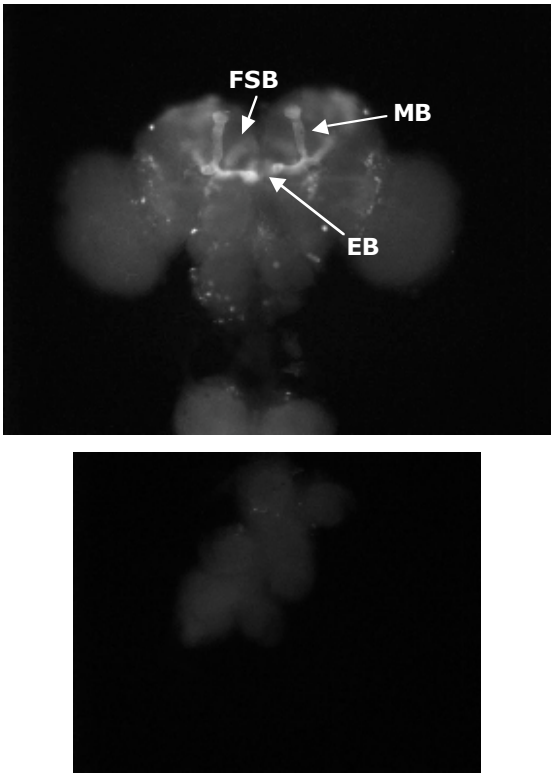
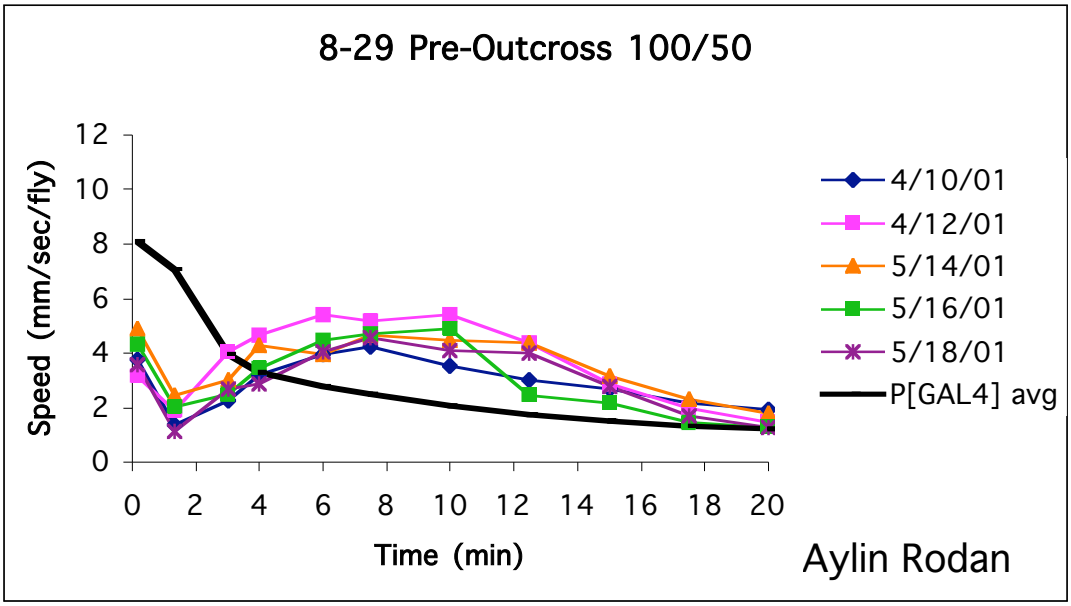

FSB
MB
EB

Figure 4

A


8-29 Pre-Outcross 100/50
Speed (mm/sec/fly)
Time (min)
4/10/01
4/12/01
5/14/01
5/16/01
5/18/01
P[GAL4] avg
Aylin Rodan

B

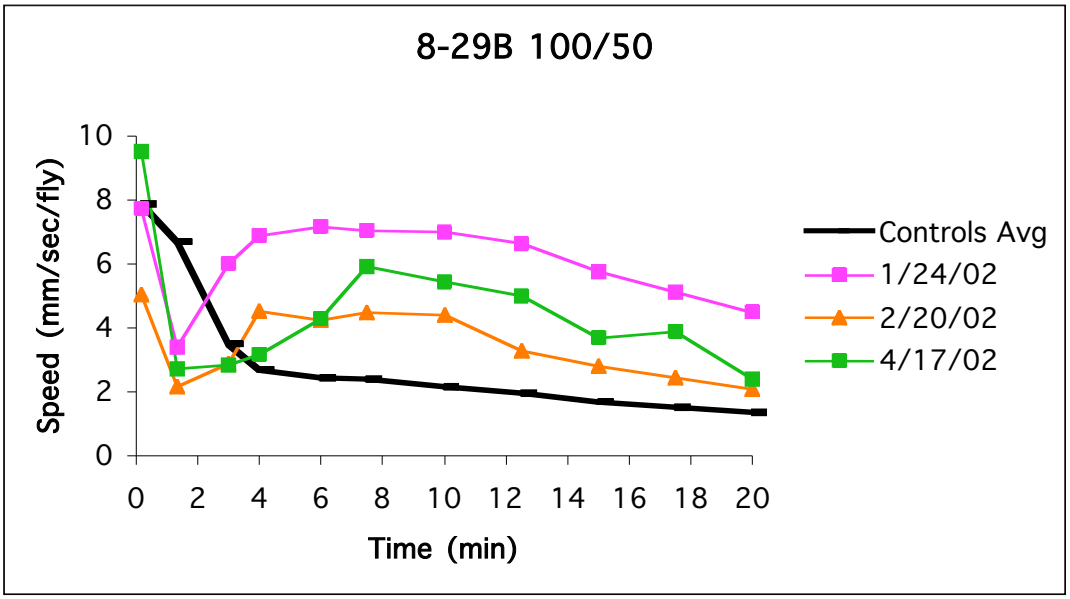
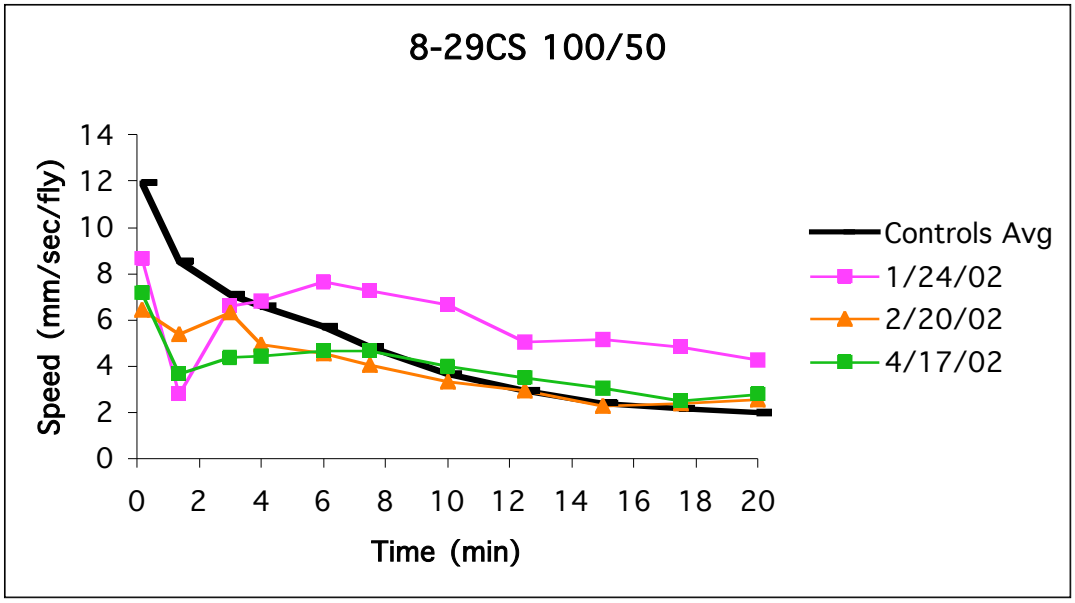

8-29B 100/50
Speed (mm/sec/fly)
Time (min)
Controls Avg
1/24/02
2/20/02
4/17/02

Figure 4

C


8-29CS 100/50
Speed (mm/sec/fly)
Time (min)
Controls Avg
1/24/02
2/20/02
4/17/02

D

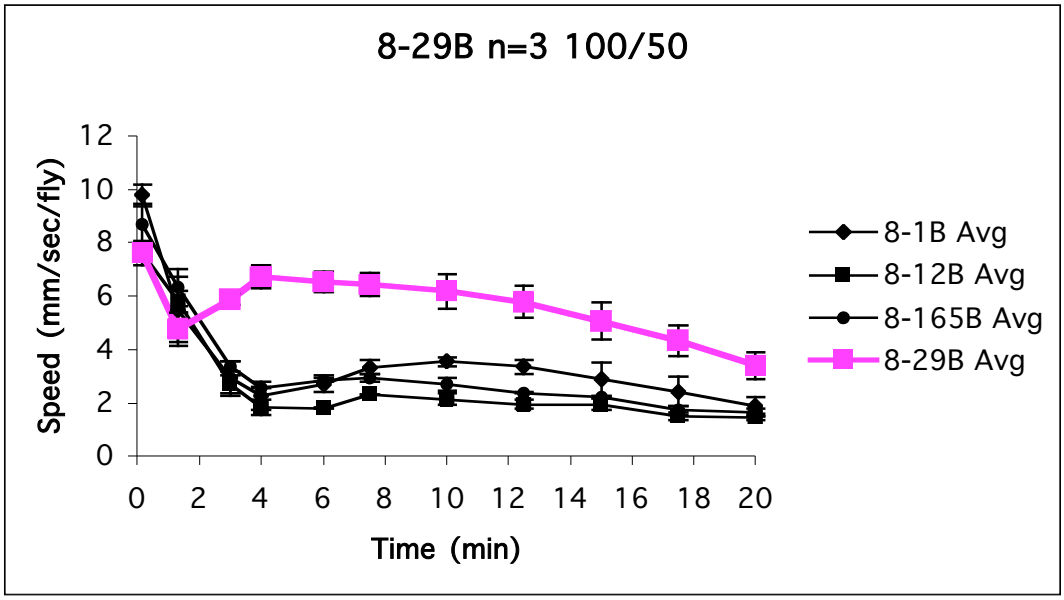
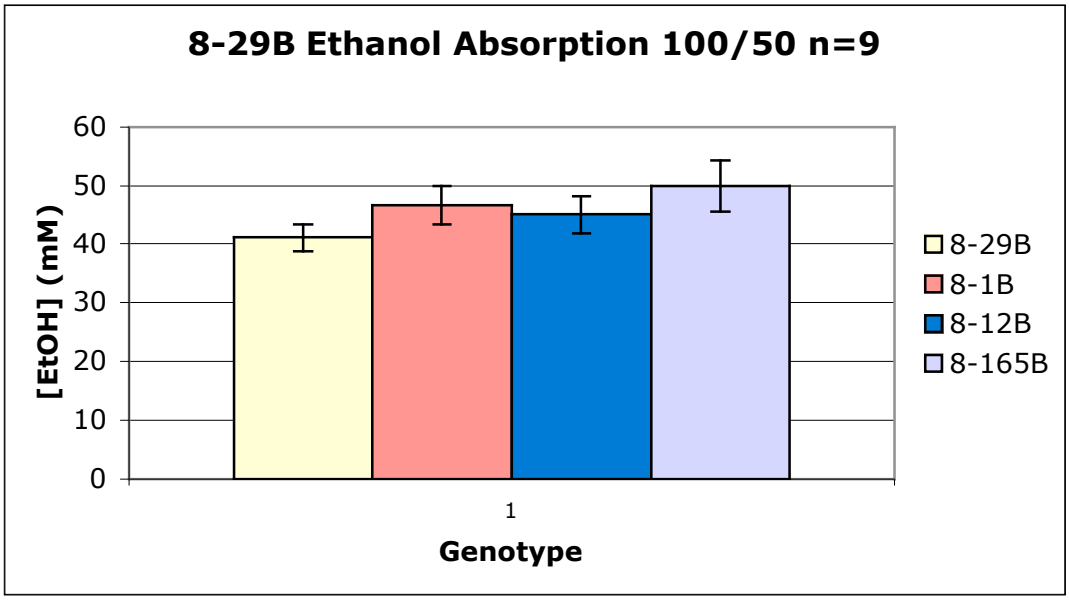
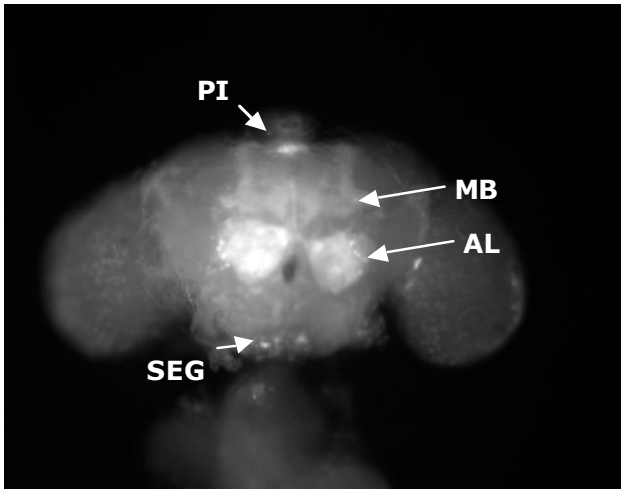

8-29B n=3 100/50
Speed (mm/sec/fly)
Time (min)
8-1B Avg
8-12B Avg
8-165B Avg
8-29B Avg

Figure 4

E


8-29B Ethanol Absorption 100/50 n=9
[EtOH] (mM)
60
50
40
30
20
10
0
1
Genotype
8-29B
8-1B
8-12B
8-165B

F


PI
MB
AL
SEG

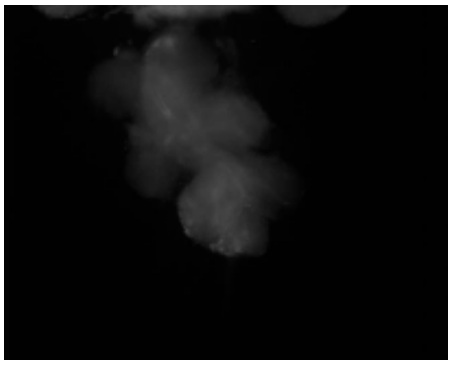
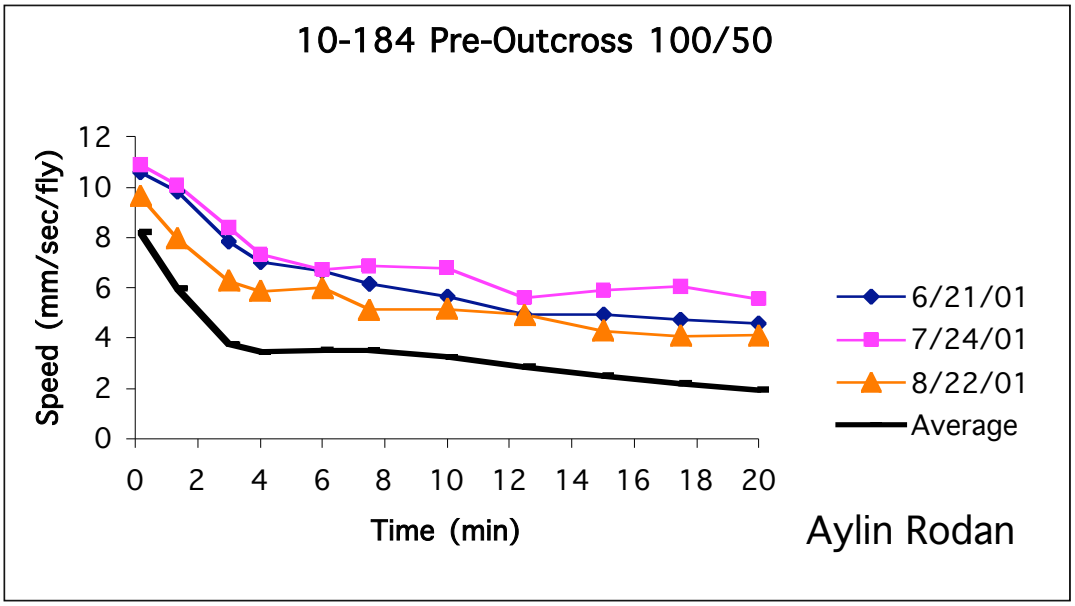


Figure 5

A


10-184 Pre-Outcross 100/50
Speed (mm/sec/fly)
Time (min)
6/21/01
7/24/01
8/22/01
Average
Aylin Rodan

B

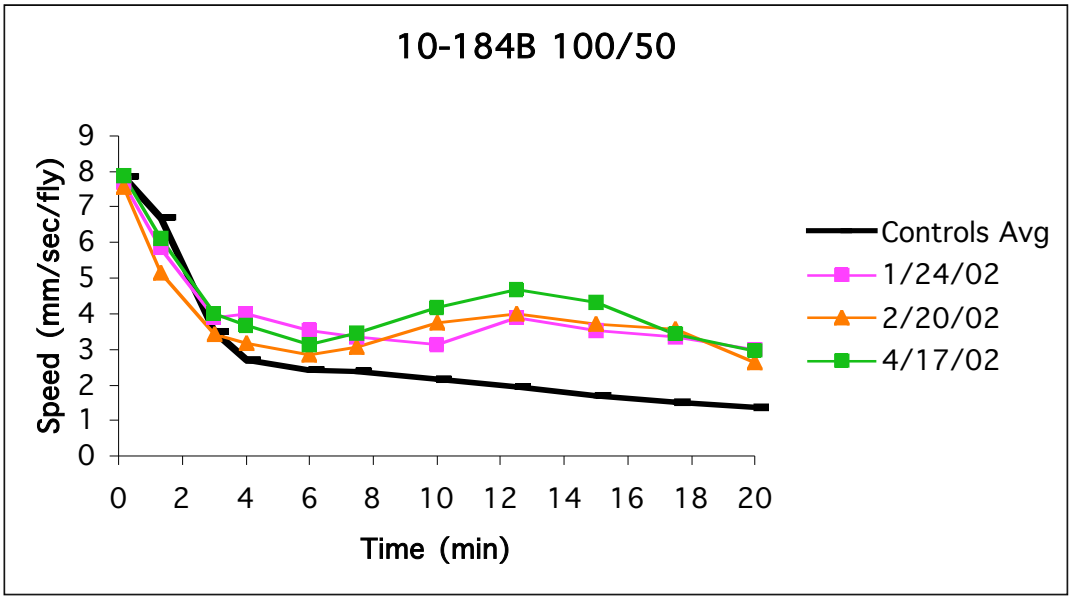
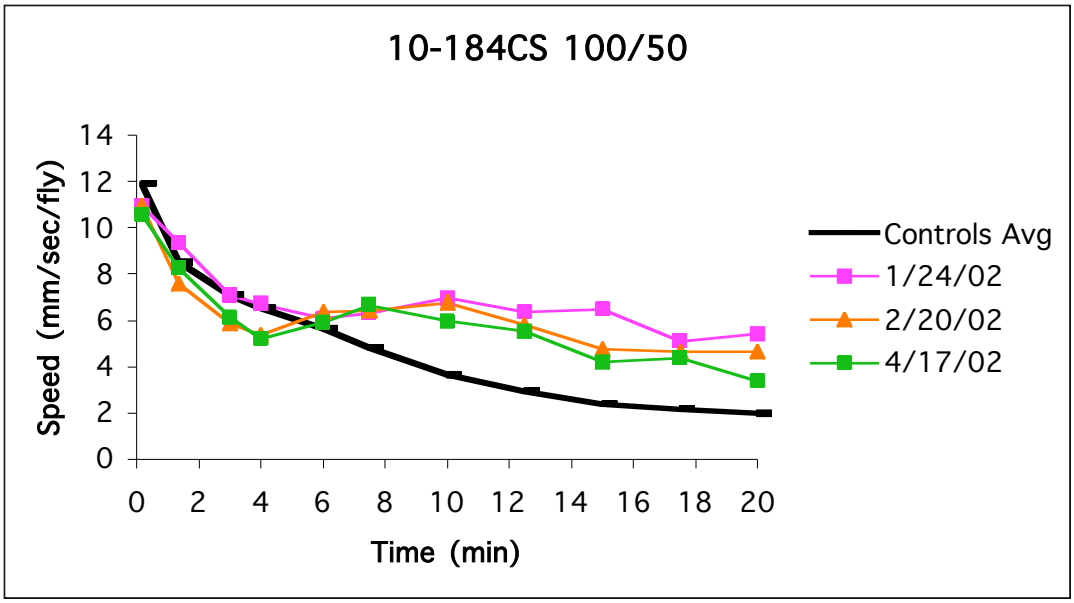

10-184B 100/50
Speed (mm/sec/fly)
Time (min)
Controls Avg
1/24/02
2/20/02
4/17/02

Figure 5

C


10-184CS 100/50
Speed (mm/sec/fly)
Time (min)
Controls Avg
1/24/02
2/20/02
4/17/02

D

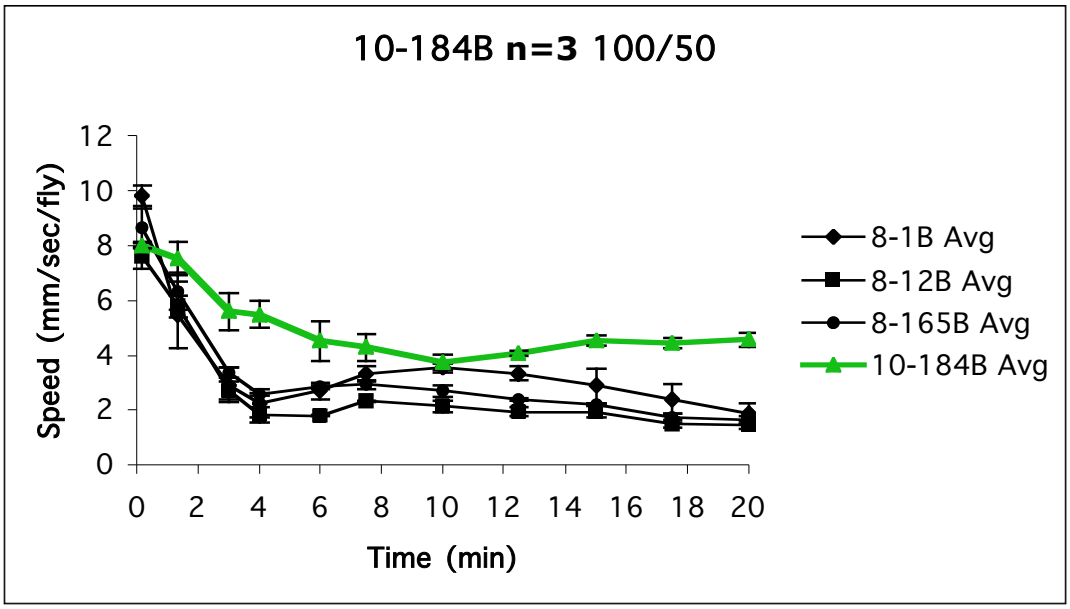
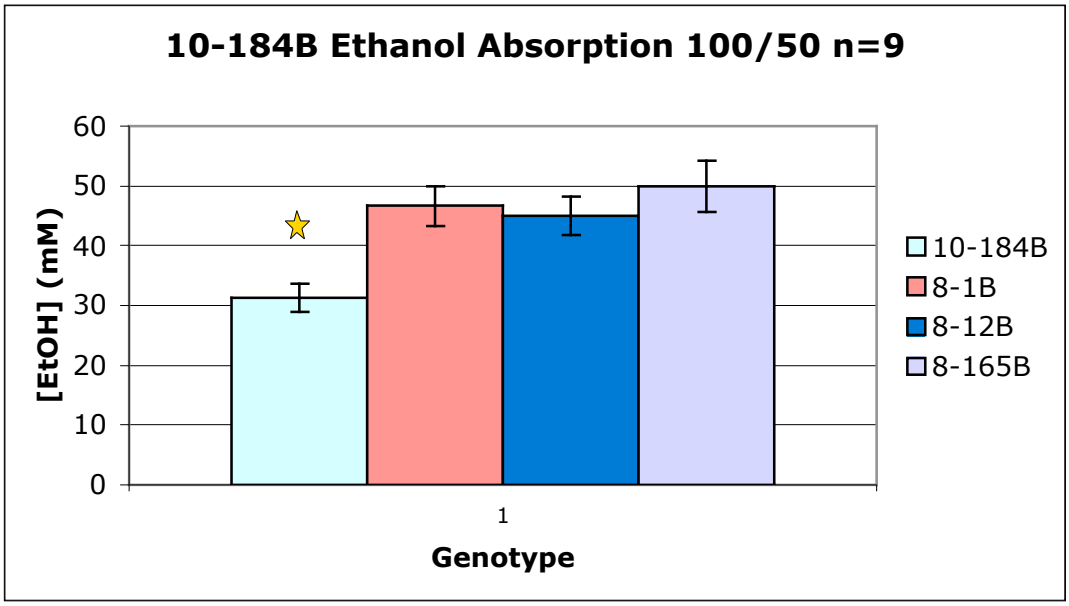

10-184B n=3 100/50
Speed (mm/sec/fly)
Time (min)
8-1B Avg
8-12B Avg
8-165B Avg
10-184B Avg

Figure 5

E


10-184B Ethanol Absorption 100/50 n=9
[EtOH] (mM)
60
50
40
30
20
10
0
1
Genotype
10-184B
8-1B
8-12B
8-165B

F

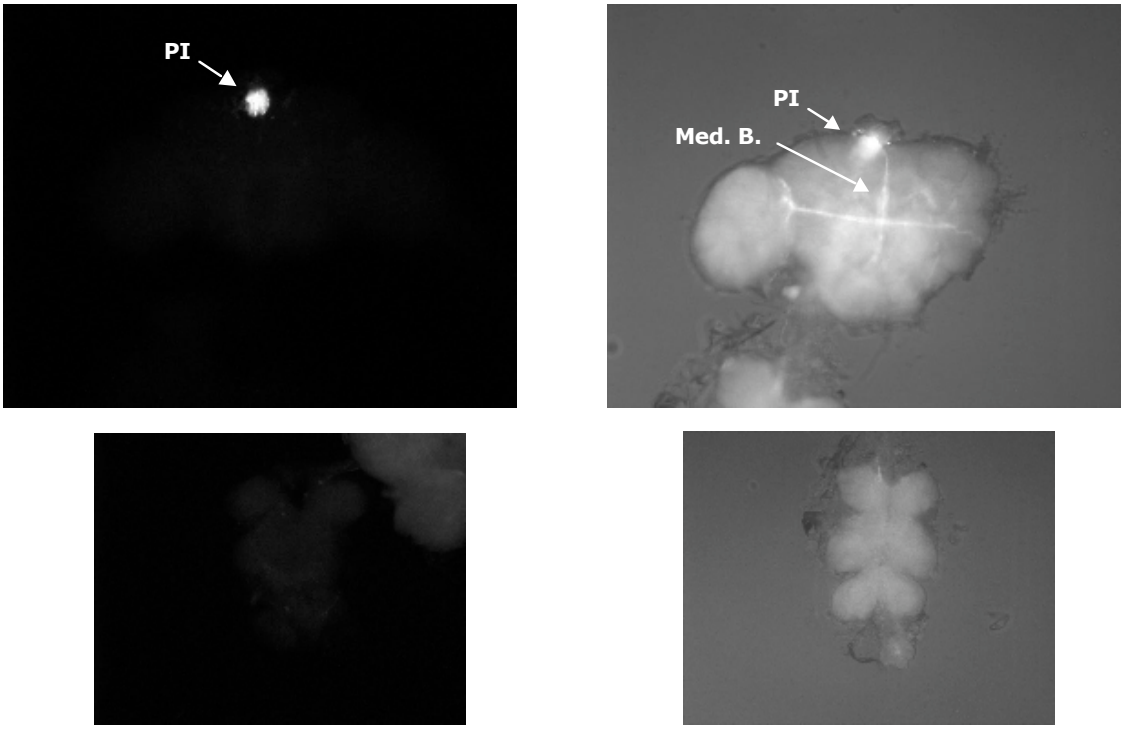
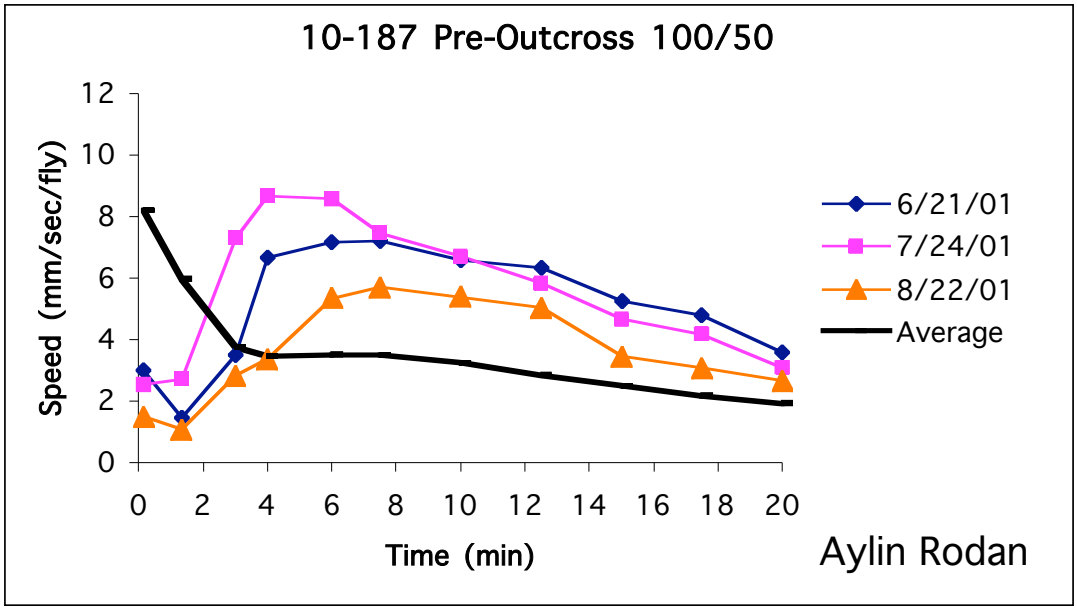

PI
PI
Med. B.

Figure 6

A


10-187 Pre-Outcross 100/50
Speed (mm/sec/fly)
Time (min)
6/21/01
7/24/01
8/22/01
Average
Aylin Rodan

B

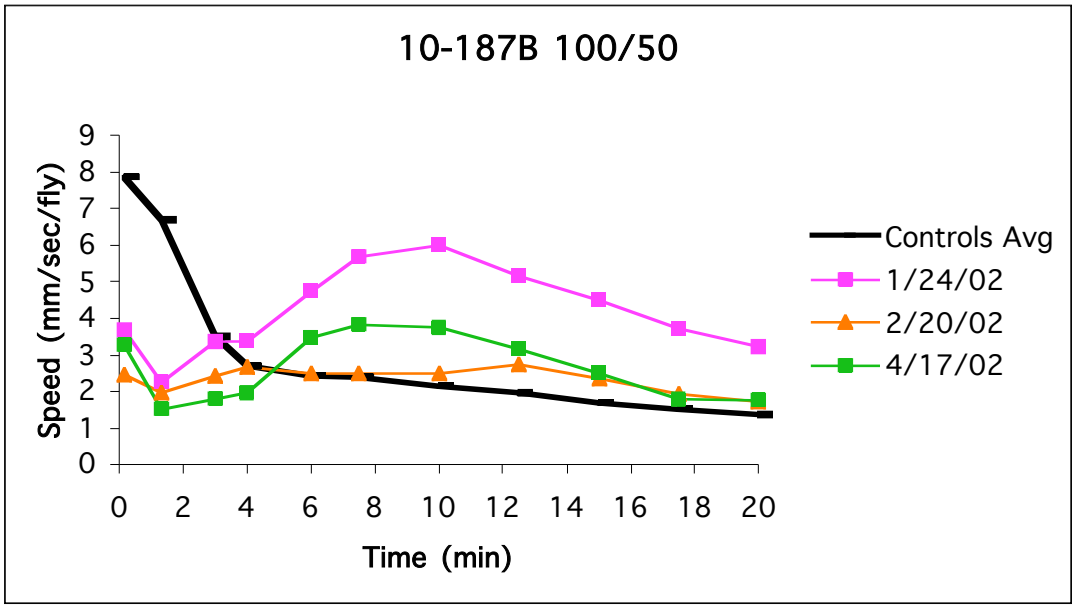
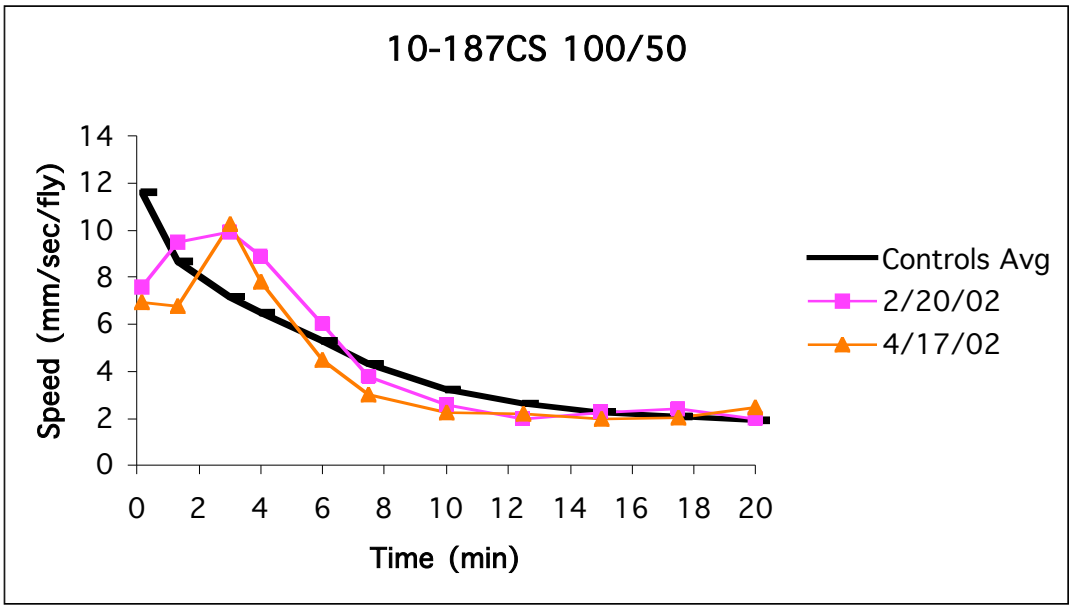

10-187B 100/50
Speed (mm/sec/fly)
Time (min)
Controls Avg
1/24/02
2/20/02
4/17/02

Figure 6

C


10-187CS 100/50
Speed (mm/sec/fly)
Time (min)
Controls Avg
2/20/02
4/17/02

D

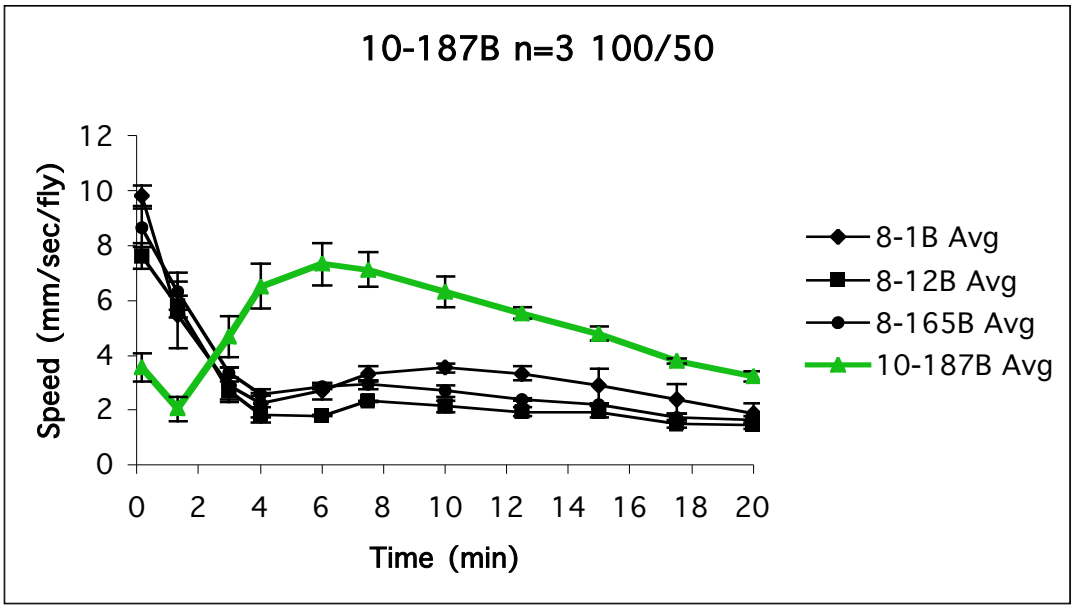
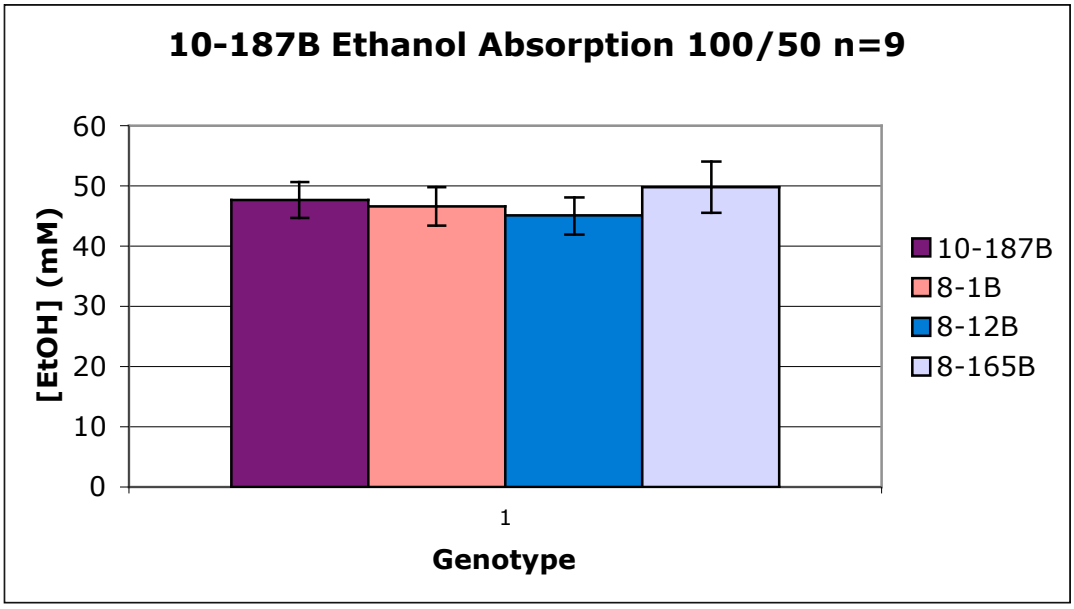

10-187B n=3 100/50
Speed (mm/sec/fly)
Time (min)
8-1B Avg
8-12B Avg
8-165B Avg
10-187B Avg

Figure 6

E


10-187B Ethanol Absorption 100/50 n=9
[EtOH] (mM)
60
50
40
30
20
10
0
1
Genotype
10-187B
8-1B
8-12B
8-165B

F

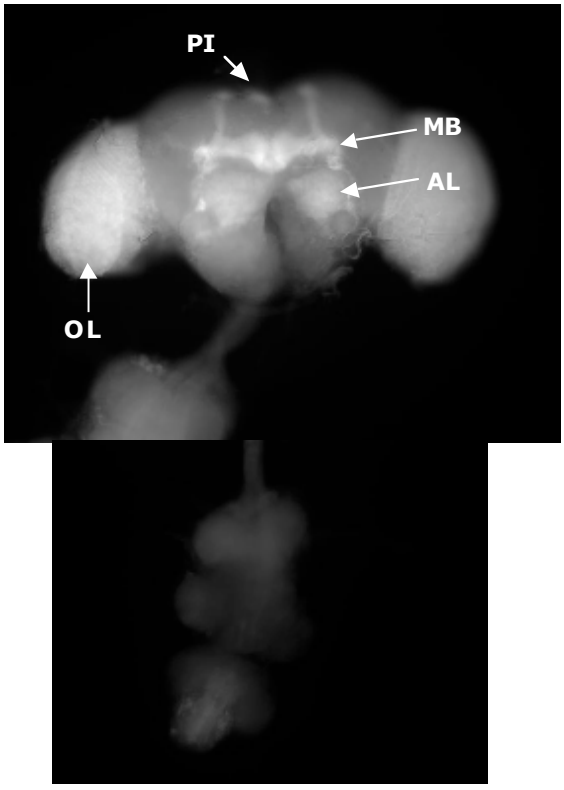
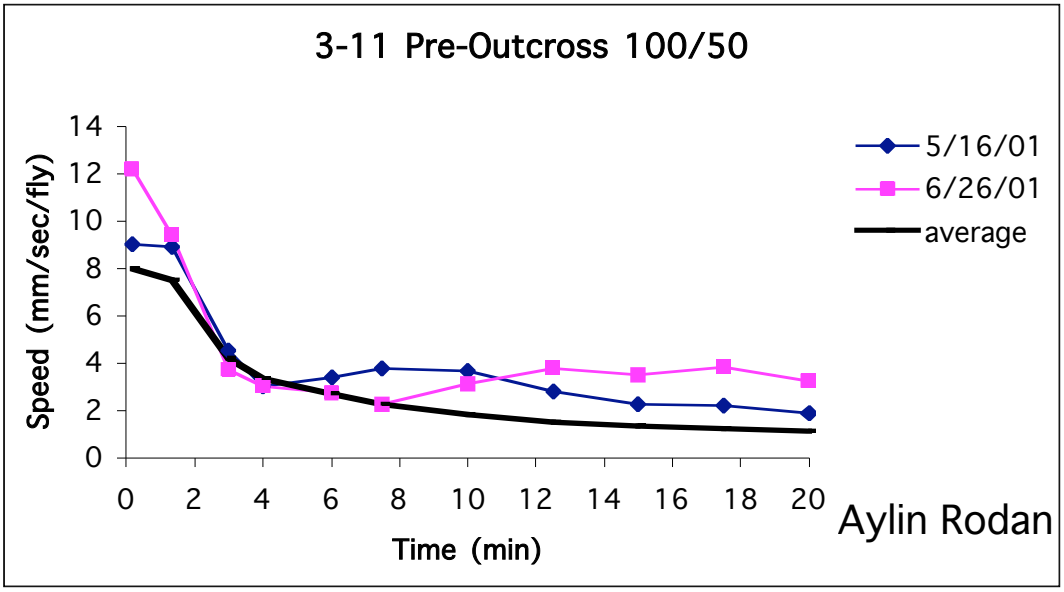

PI
MB
AL
OL

Figure 7

A


3-11 Pre-Outcross 100/50
Speed (mm/sec/fly)
Time (min)
5/16/01
6/26/01
average
Aylin Rodan

B

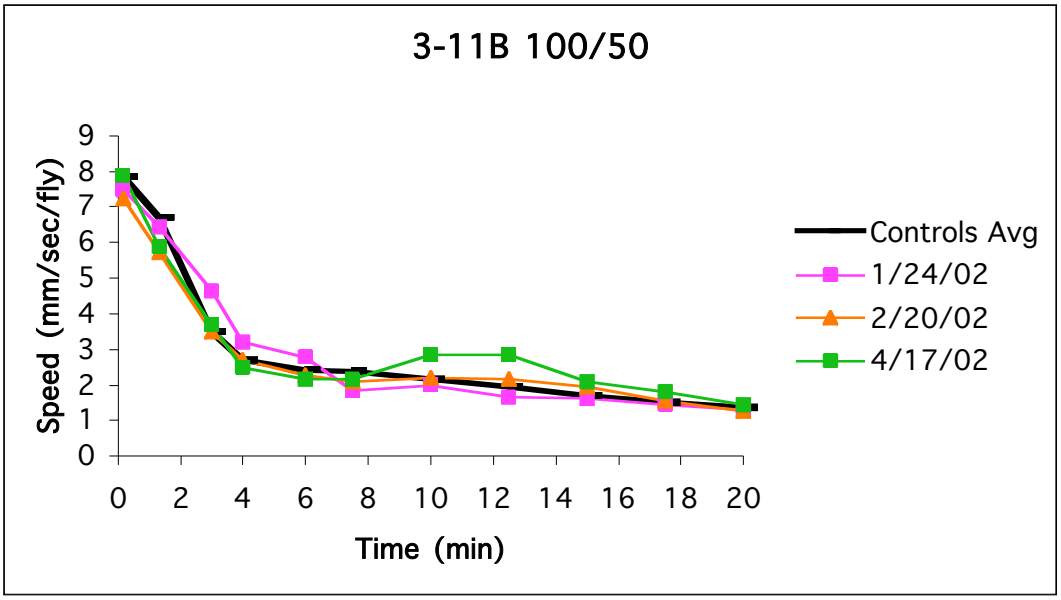
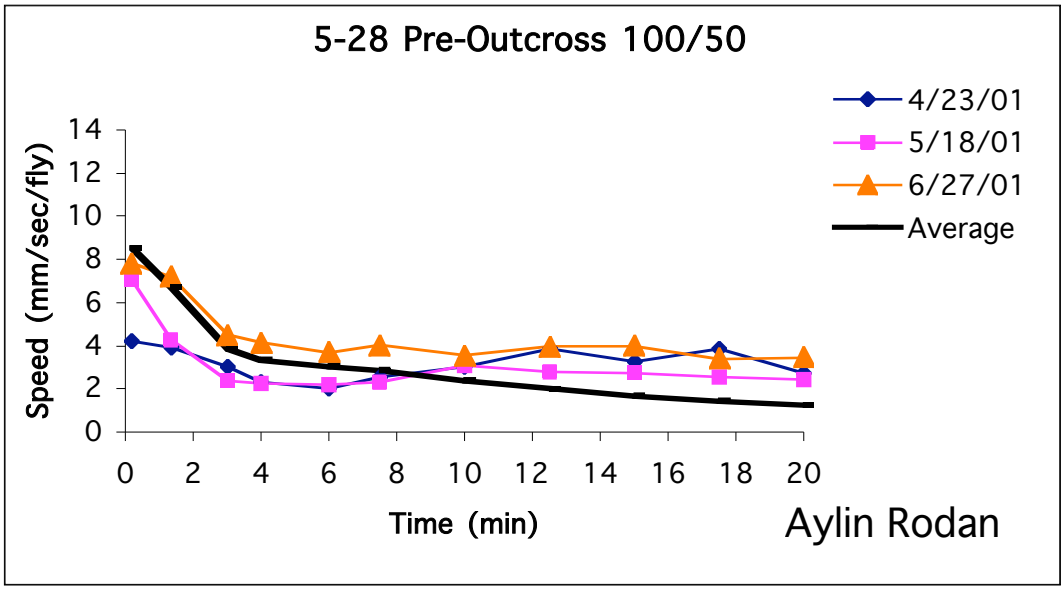

3-11B 100/50
Speed (mm/sec/fly)
Time (min)
Controls Avg
1/24/02
2/20/02
4/17/02

Figure 7

C


5-28 Pre-Outcross 100/50
Speed (mm/sec/fly)
Time (min)
4/23/01
5/18/01
6/27/01
Average
Aylin Rodan

D

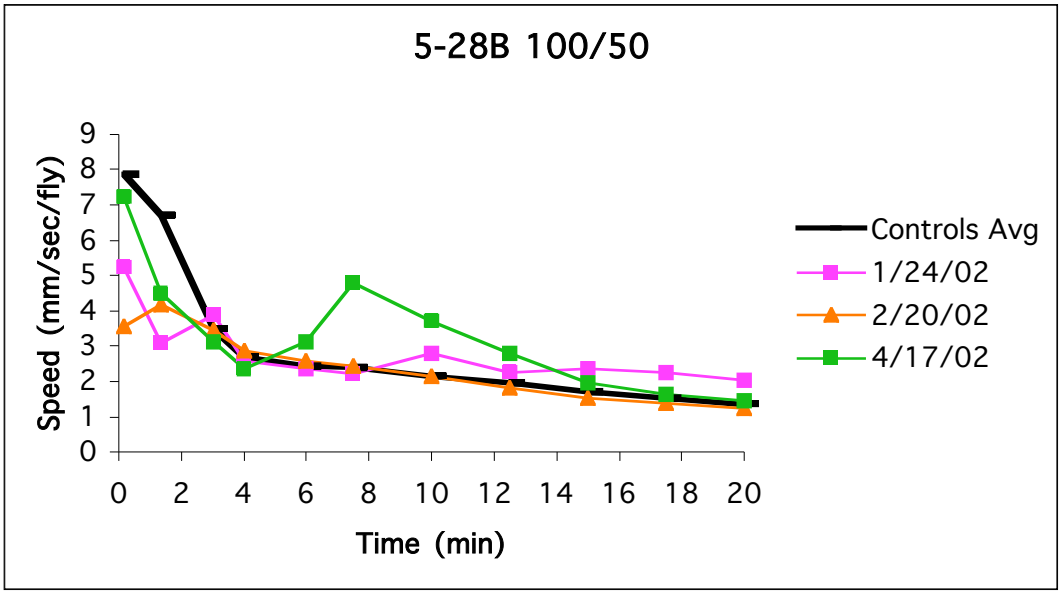
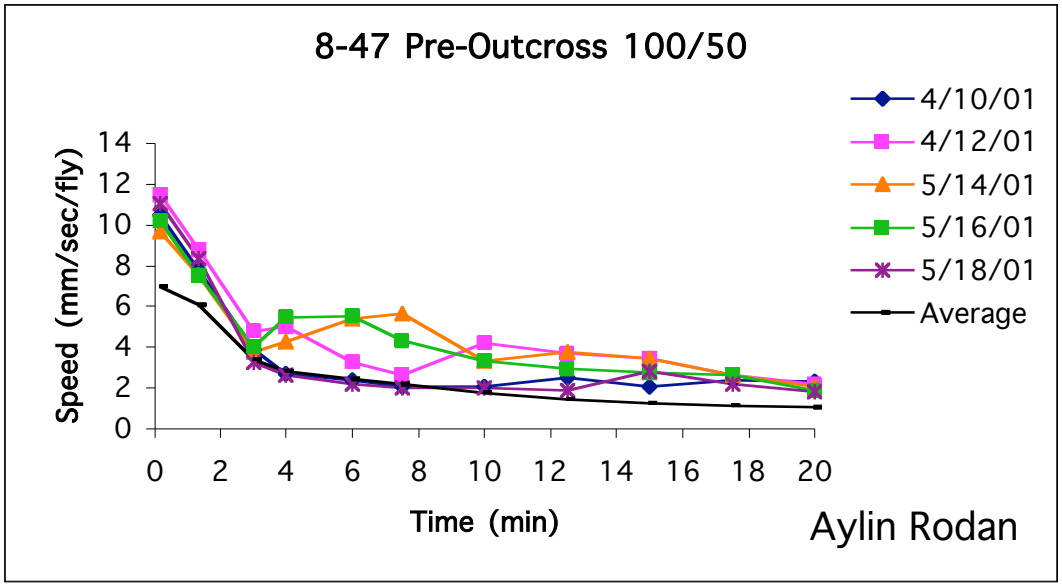

5-28B 100/50
Speed (mm/sec/fly)
Time (min)
Controls Avg
1/24/02
2/20/02
4/17/02

Figure 7

E


8-47 Pre-Outcross 100/50
Speed (mm/sec/fly)
Time (min)
4/10/01
4/12/01
5/14/01
5/16/01
5/18/01
Average
Aylin Rodan

F

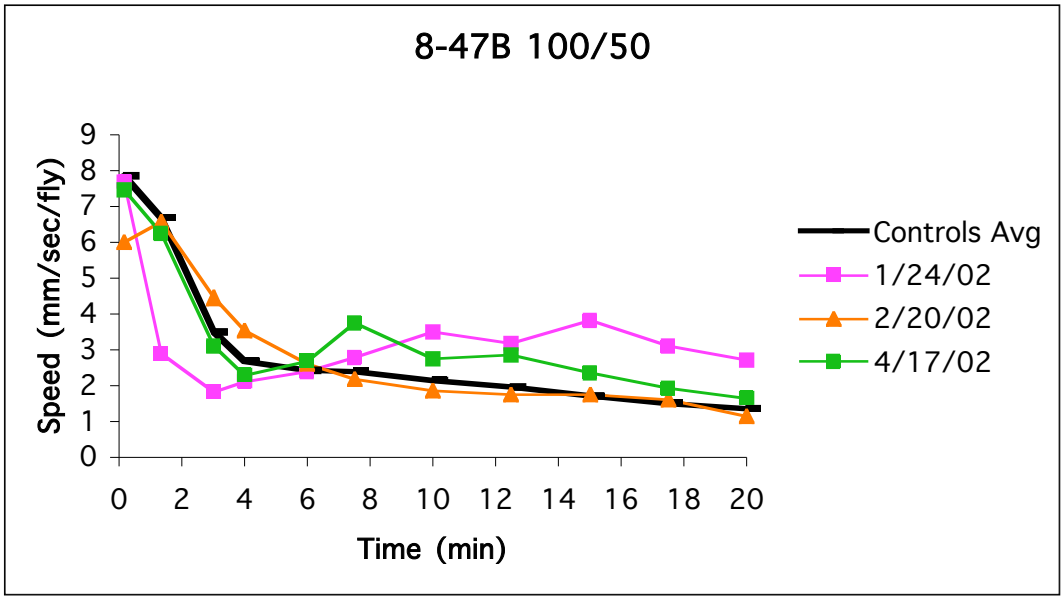
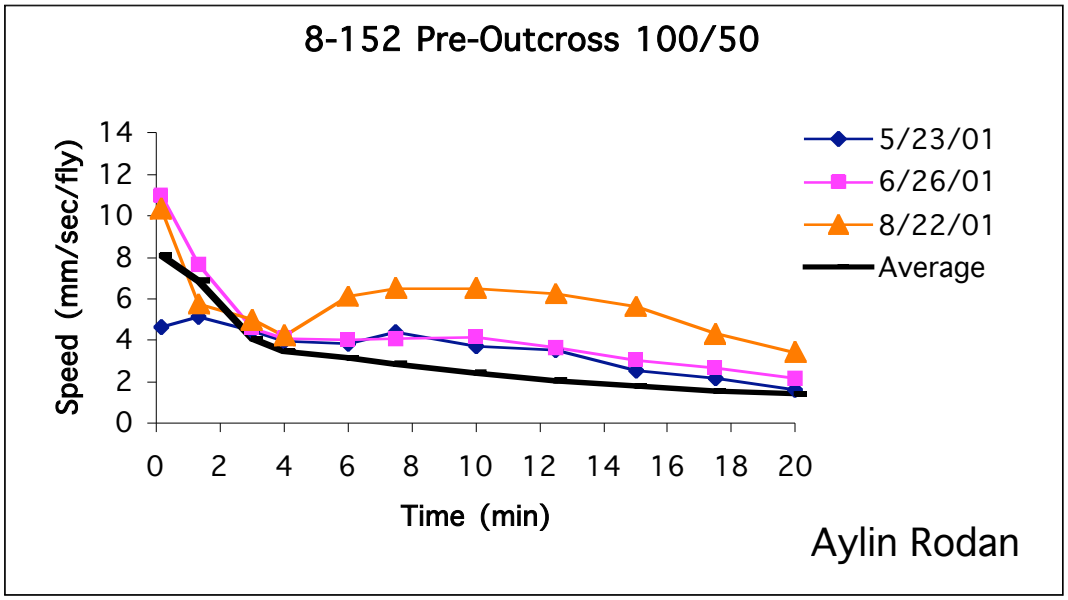

8-47B 100/50
Speed (mm/sec/fly)
Time (min)
Controls Avg
1/24/02
2/20/02
4/17/02

Figure 7

G


8-152 Pre-Outcross 100/50
Speed (mm/sec/fly)
Time (min)
5/23/01
6/26/01
8/22/01
Average
Aylin Rodan

H

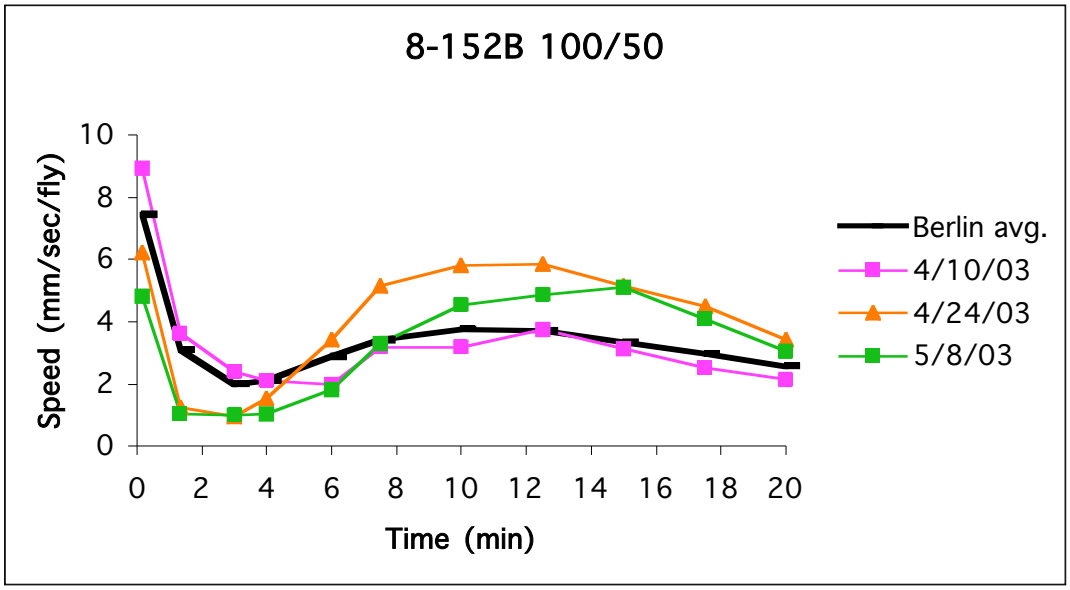
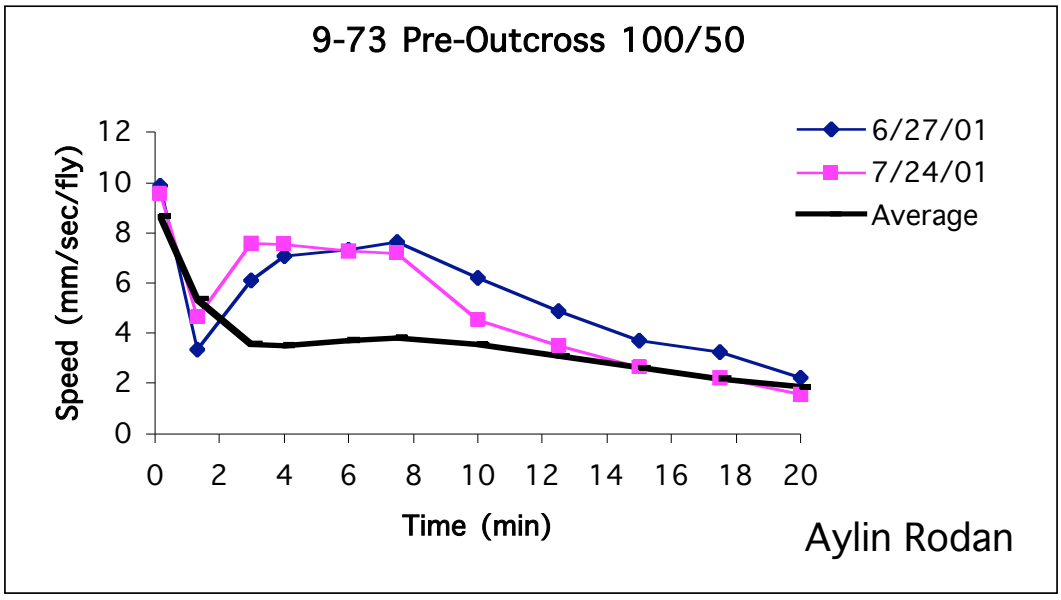

8-152B 100/50
Speed (mm/sec/fly)
Time (min)
Berlin avg.
4/10/03
4/24/03
5/8/03

Figure 7

I


9-73 Pre-Outcross 100/50
Speed (mm/sec/fly)
Time (min)
6/27/01
7/24/01
Average
Aylin Rodan

J

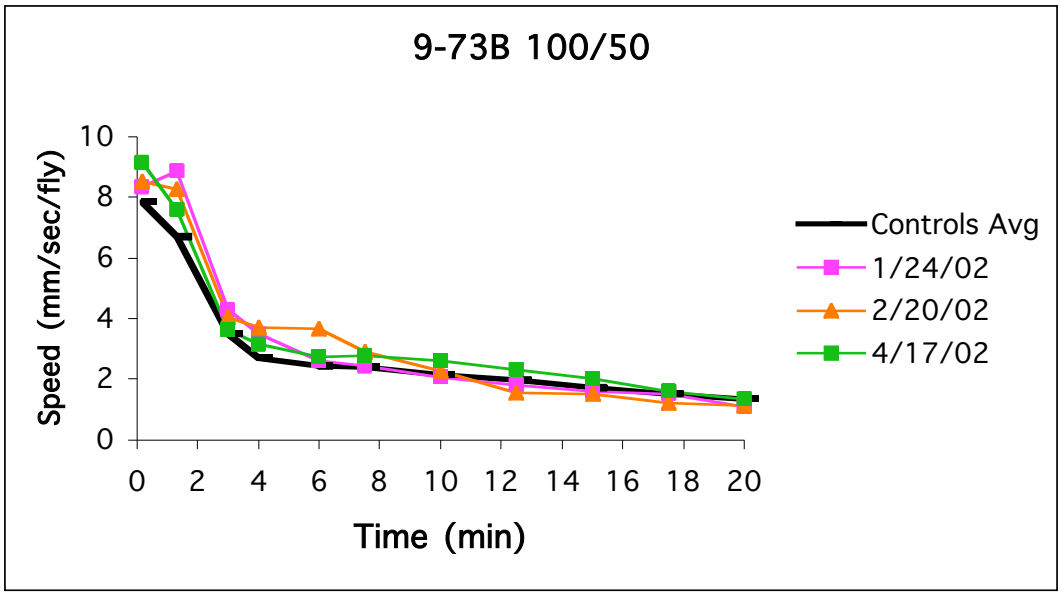

9-73B 100/50
Speed (mm/sec/fly)
Time (min)
Controls Avg
1/24/02
2/20/02
4/17/02

Figure 8

A

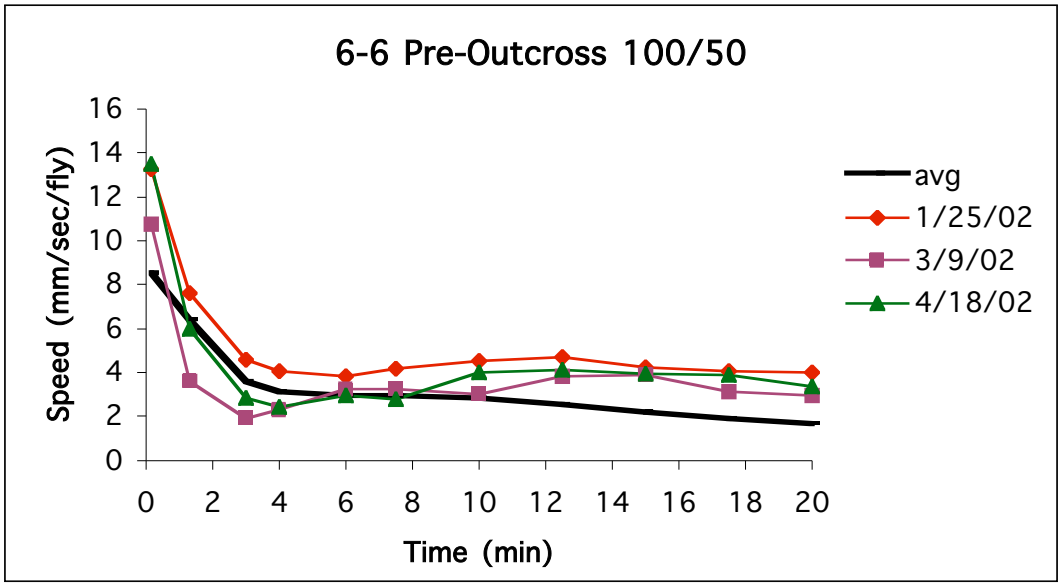
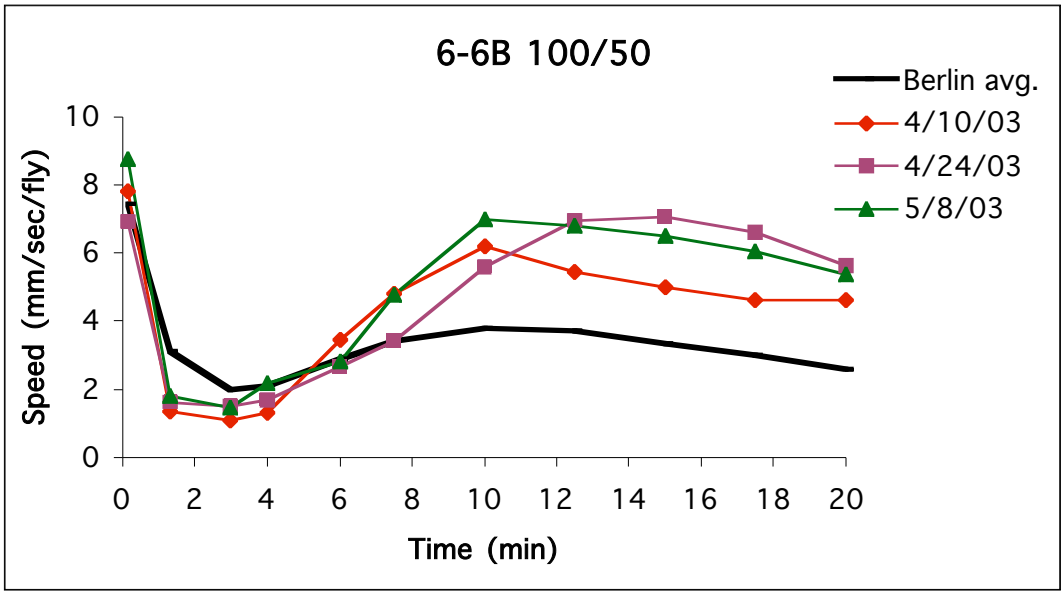

6-6 Pre-Outcross 100/50
Speed (mm/sec/fly)
16
14
12
10
8
6
4
2
0
0
2
4
6
8
10
12
14
16
18
20
Time (min)
avg
1/25/02
3/9/02
4/18/02

Figure 8

B


6-6B 100/50
Berlin avg.
4/10/03
4/24/03
5/8/03
Speed (mm/sec/fly)
Time (min)
0
2
4
6
8
10
12
14
16
18
20

C

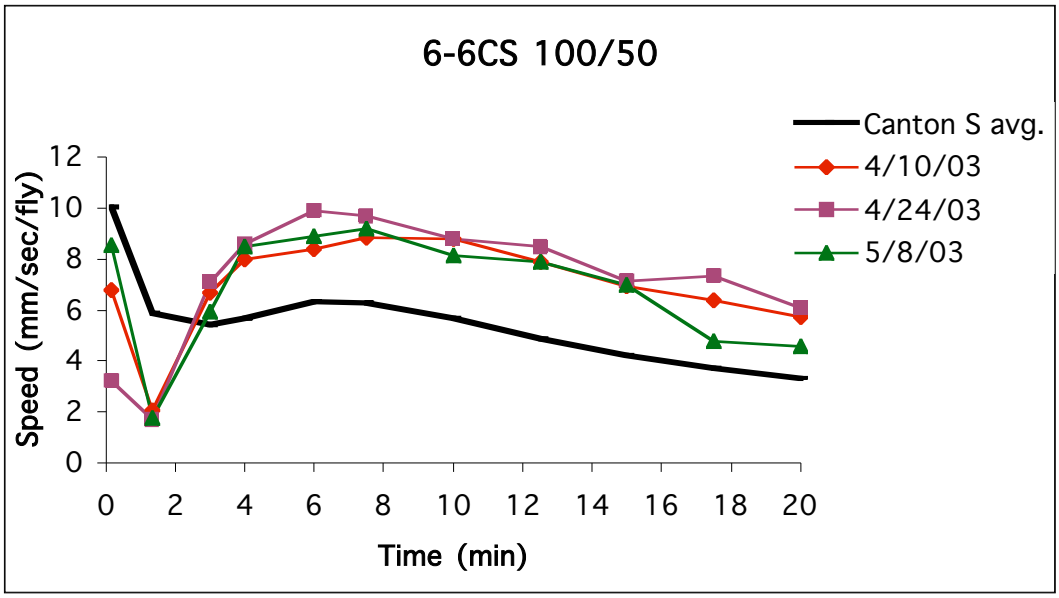
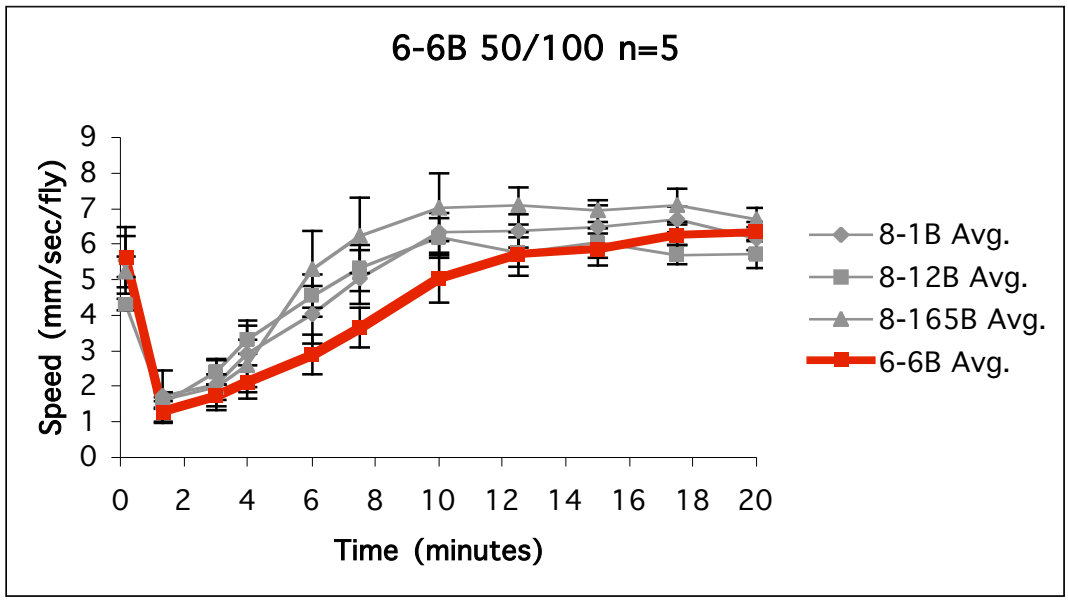

6-6CS 100/50
Canton S avg.
4/10/03
4/24/03
5/8/03
Speed (mm/sec/fly)
Time (min)
0
2
4
6
8
10
12
14
16
18
20

Figure 8

D


6-6B 50/100 n=5
Speed (mm/sec/fly)
Time (minutes)
8-1B Avg.
8-12B Avg.
8-165B Avg.
6-6B Avg.

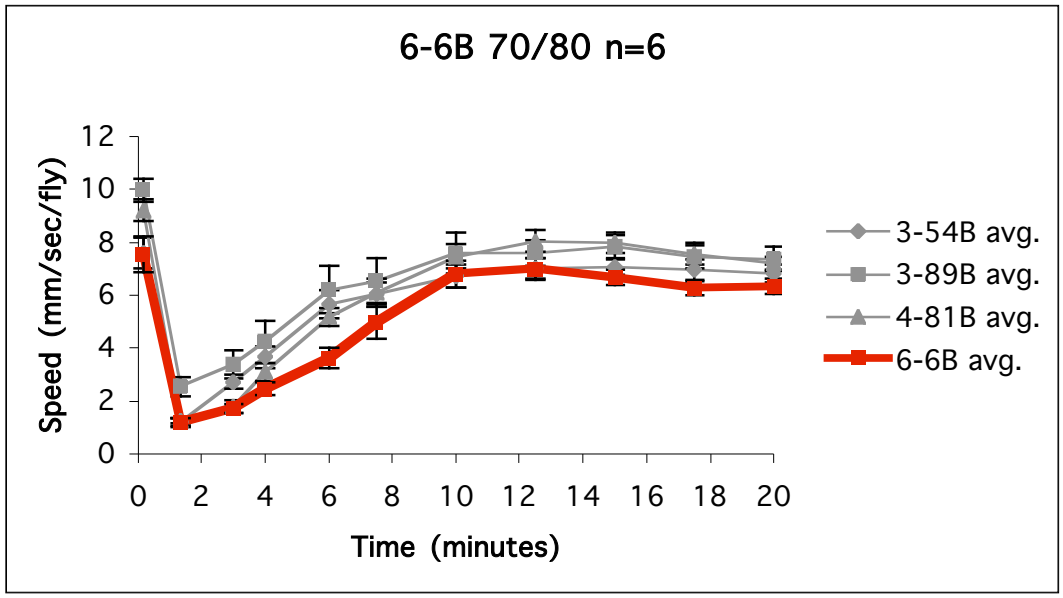
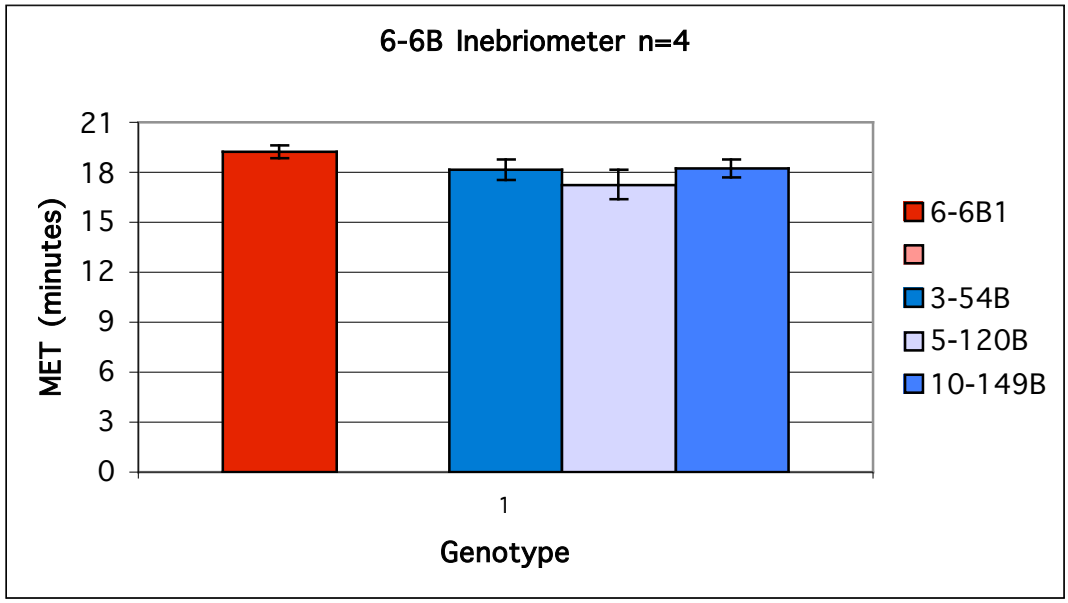

6-6B 70/80 n=6
Speed (mm/sec/fly)
Time (minutes)
3-54B avg.
3-89B avg.
4-81B avg.
6-6B avg.

Figure 8

E


6-6B Inebriometer n=4
MET (minutes)
21
18
15
12
9
6
3
0
1
Genotype
6-6B1
3-54B
5-120B
10-149B

F

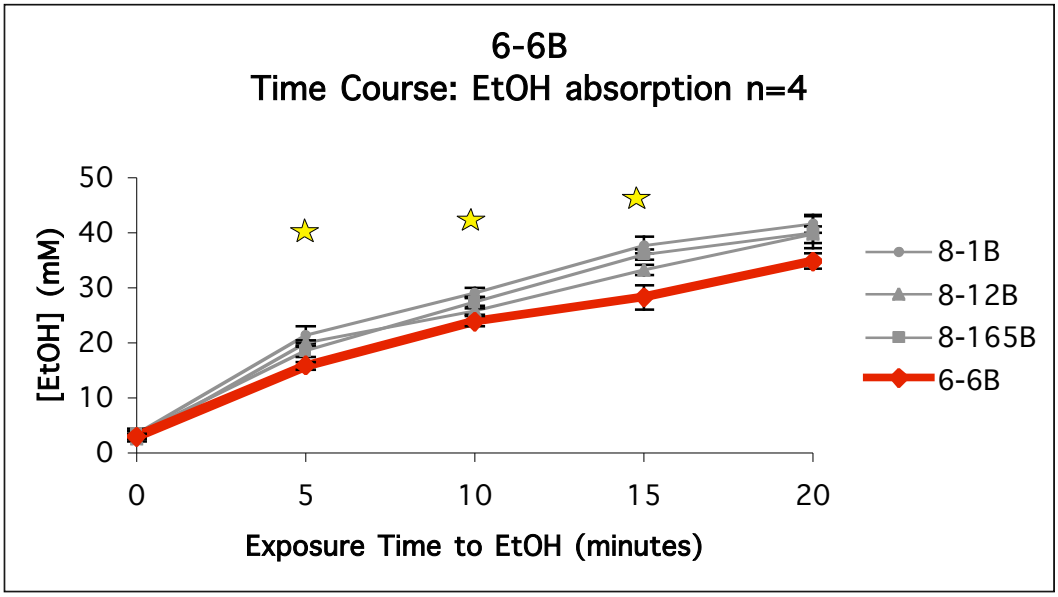

6-6B
Time Course: EtOH absorption n=4
[EtOH] (mM)
50
40
30
20
10
0
0
5
10
15
20
Exposure Time to EtOH (minutes)
8-1B
8-12B
8-165B
6-6B

Figure 8

G

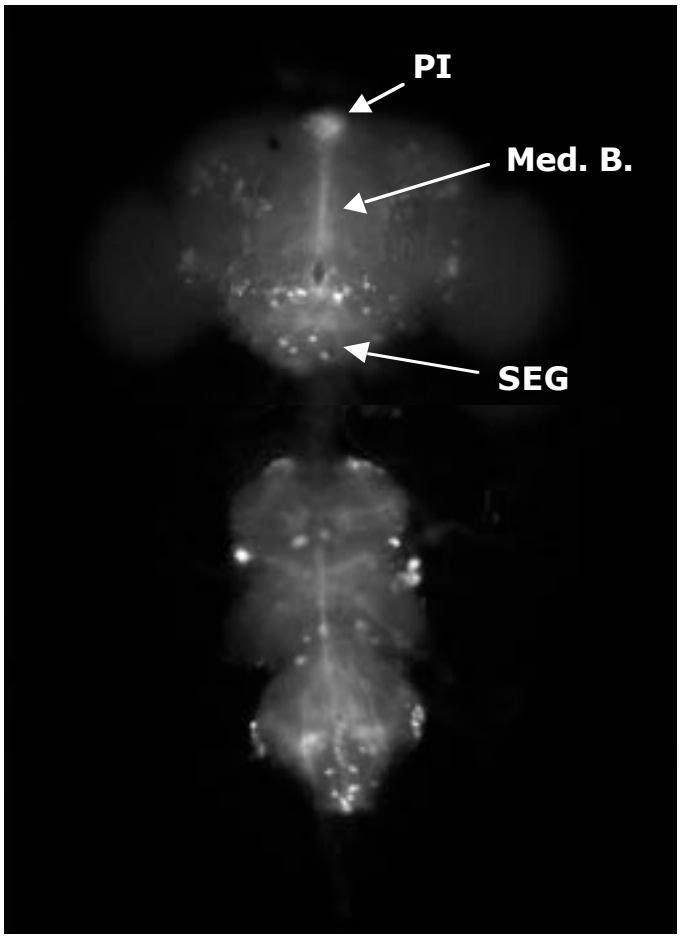


Figure 9

A

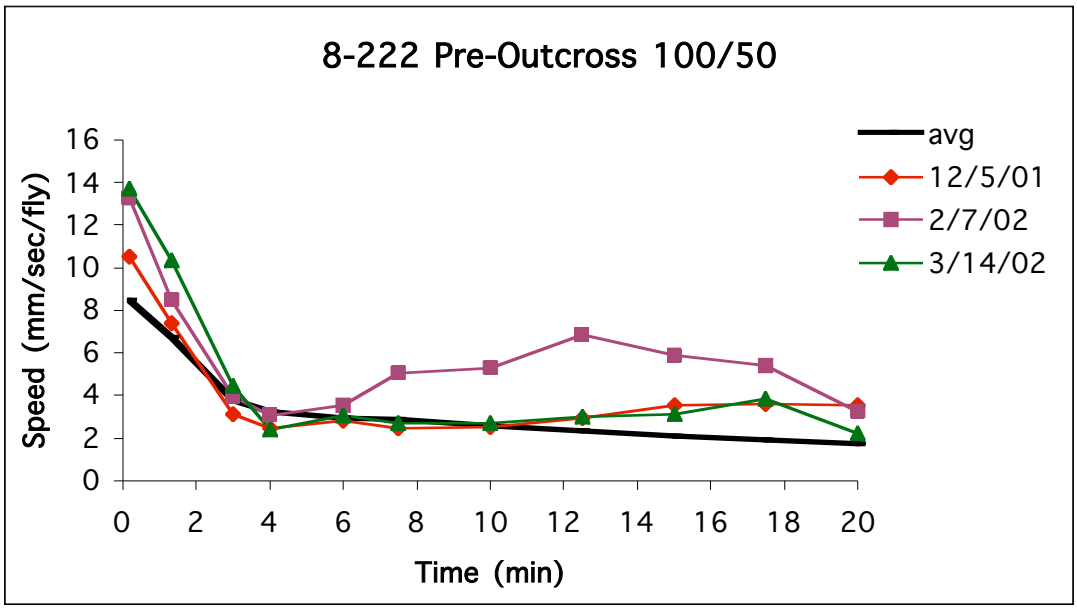
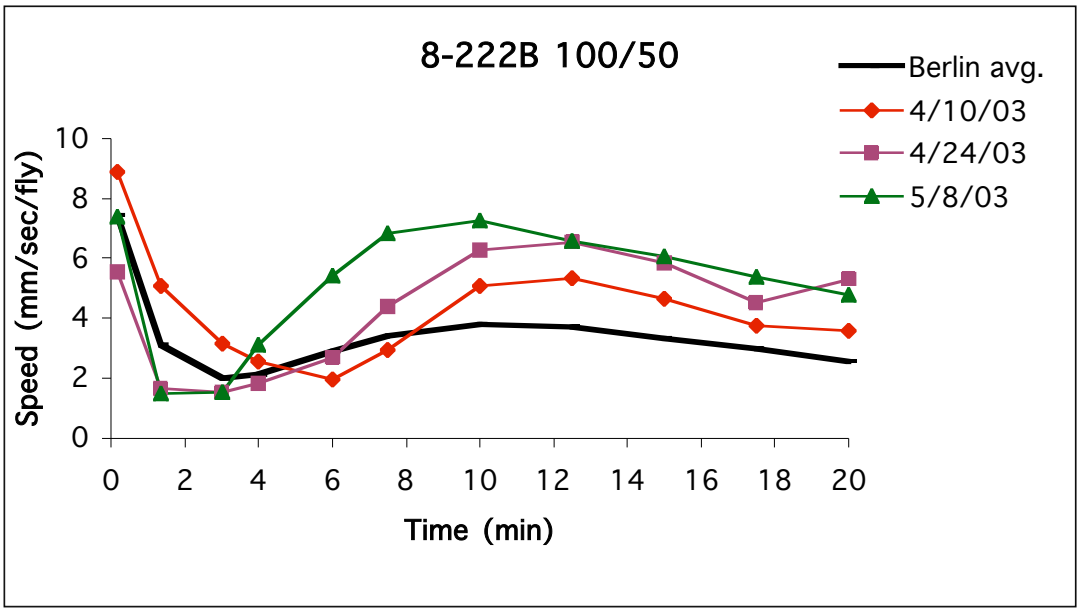

8-222 Pre-Outcross 100/50
Speed (mm/sec/fly)
Time (min)
avg
12/5/01
2/7/02
3/14/02
0
2
4
6
8
10
12
14
16
18
20

Figure 9

B


8-222B 100/50
Berlin avg.
4/10/03
4/24/03
5/8/03
Speed (mm/sec/fly)
Time (min)
0
2
4
6
8
10
12
14
16
18
20

C

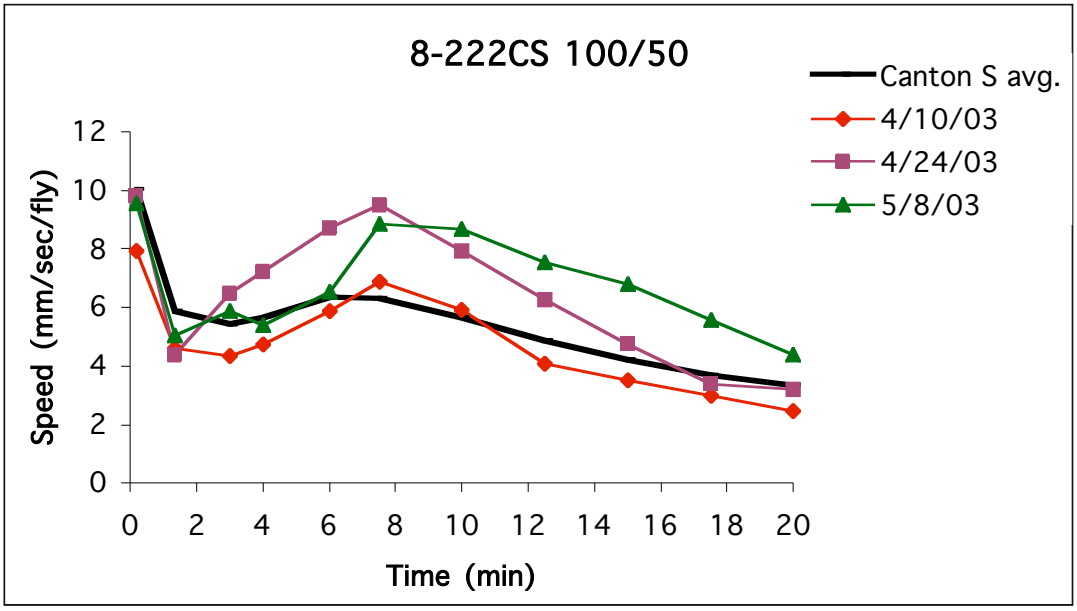
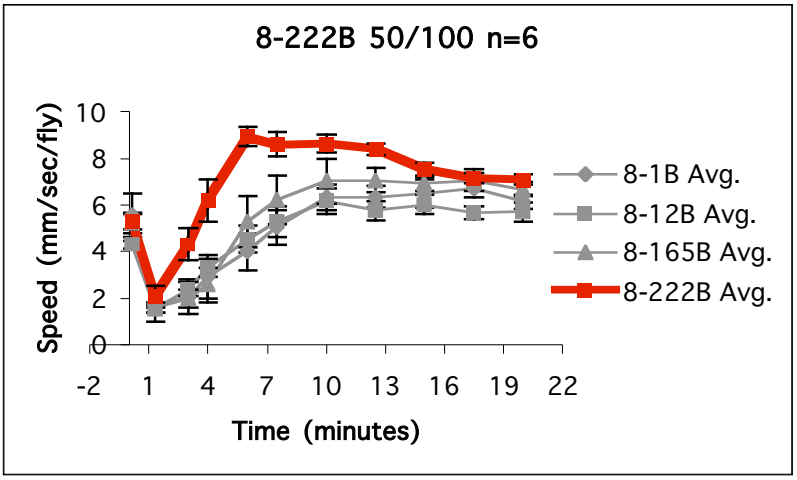
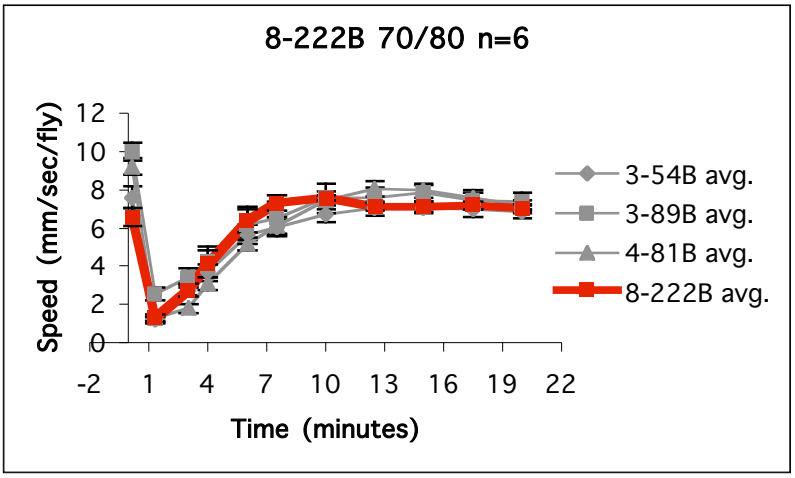

8-222CS 100/50
Canton S avg.
4/10/03
4/24/03
5/8/03
Speed (mm/sec/fly)
Time (min)
0
2
4
6
8
10
12
14
16
18
20

Figure 9

D


8-222B 50/100 n=6
Speed (mm/sec/fly)
Time (minutes)
8-1B Avg.
8-12B Avg.
8-165B Avg.
8-222B Avg.


8-222B 70/80 n=6
Speed (mm/sec/fly)
Time (minutes)
3-54B avg.
3-89B avg.
4-81B avg.
8-222B avg.

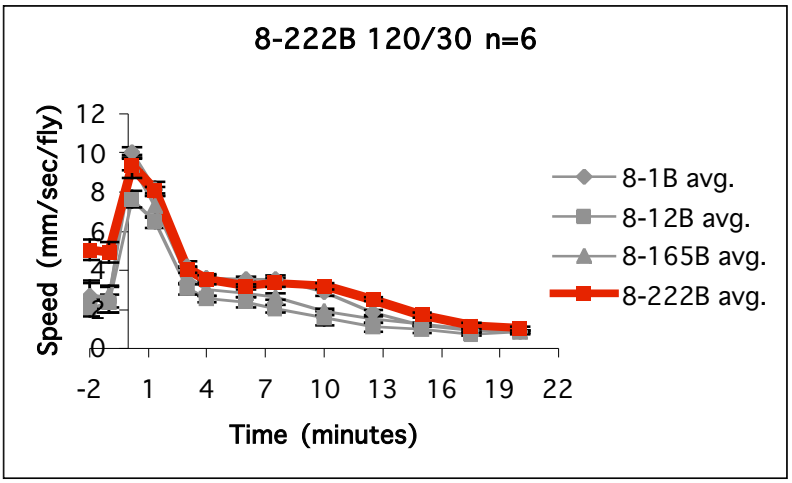
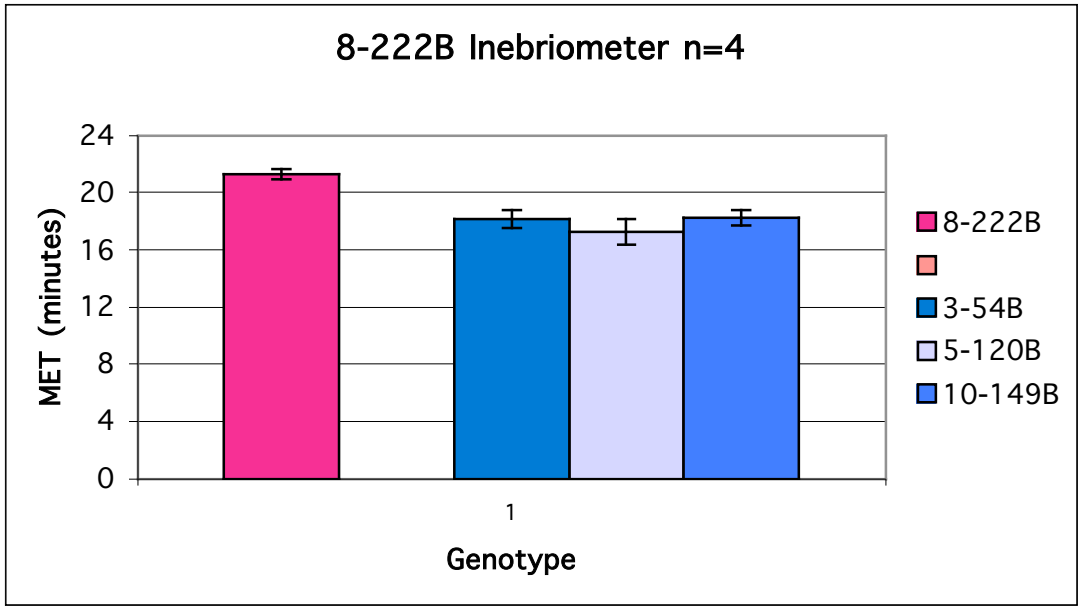

8-222B 120/30 n=6
Speed (mm/sec/fly)
Time (minutes)
8-1B avg.
8-12B avg.
8-165B avg.
8-222B avg.

Figure 9

E


8-222B Inebriometer n=4
MET (minutes)
24
20
16
12
8
4
0
1
Genotype
8-222B
3-54B
5-120B
10-149B

F

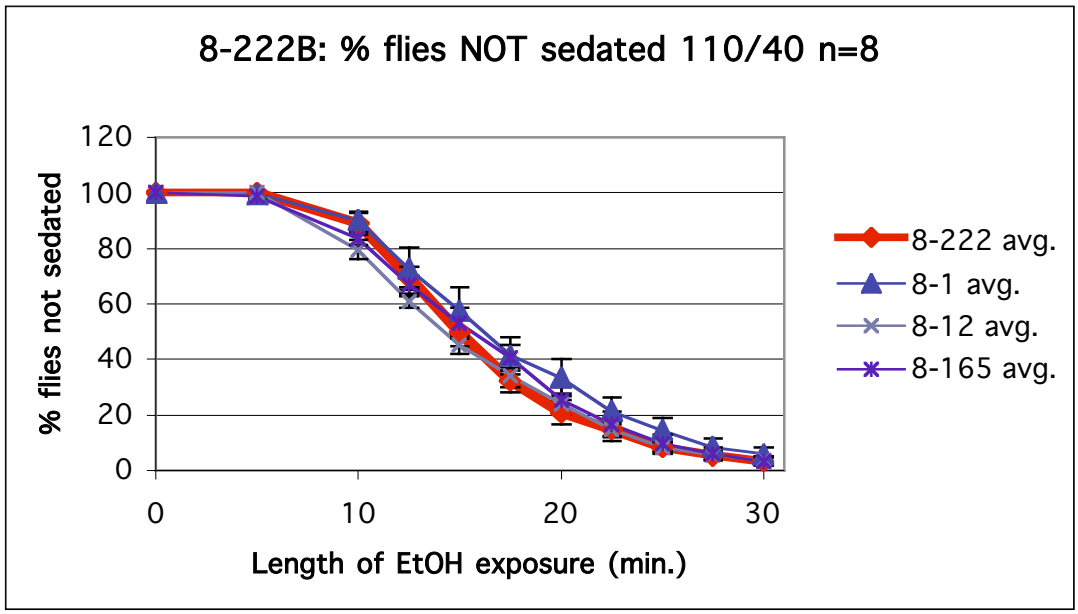
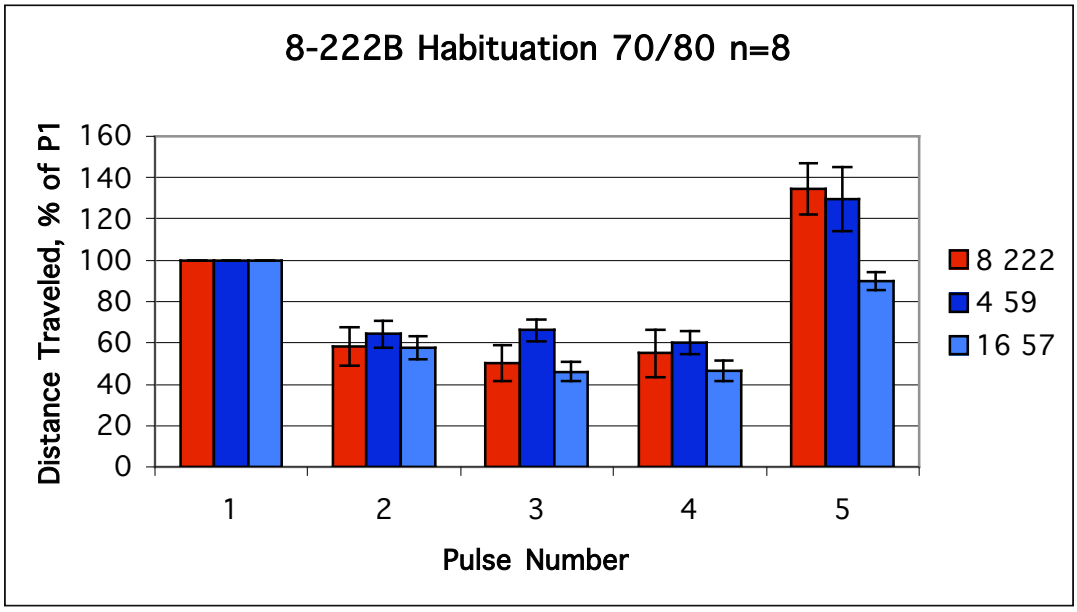

8-222B: % flies NOT sedated 110/40 n=8
% flies not sedated
120
100
80
60
40
20
0
0
10
20
30
Length of EtOH exposure (min.)
8-222 avg.
8-1 avg.
8-12 avg.
8-165 avg.

Figure 9

G


8-222B Habituation 70/80 n=8
Distance Traveled, % of P1
160
140
120
100
80
60
40
20
0
1
2
3
4
5
Pulse Number
8 222
4 59
16 57

H

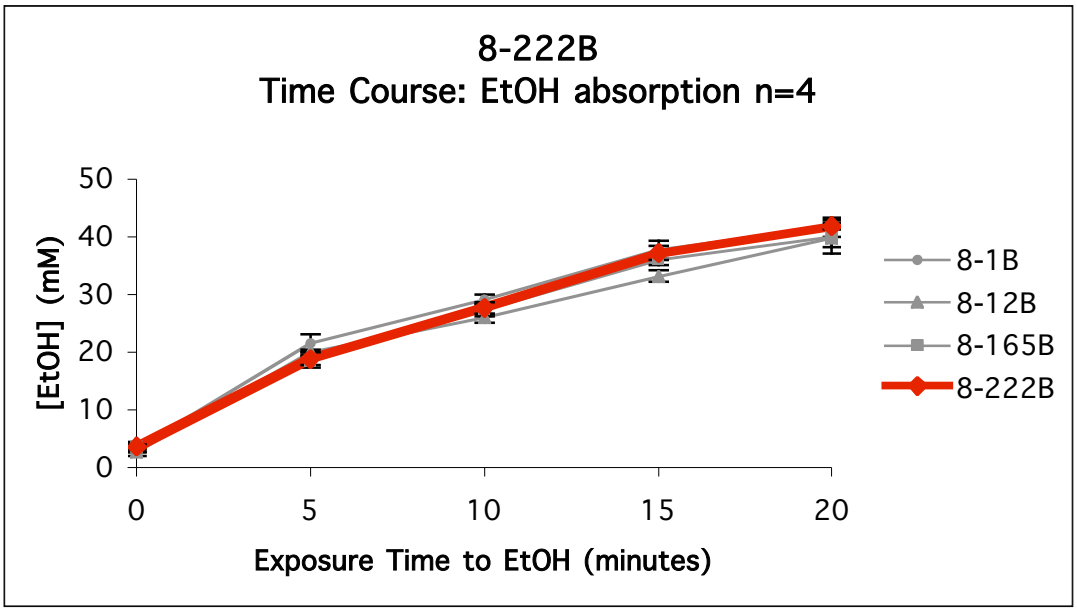

8-222B
Time Course: EtOH absorption n=4
[EtOH] (mM)
50
40
30
20
10
0
0
5
10
15
20
Exposure Time to EtOH (minutes)
8-1B
8-12B
8-165B
8-222B

Figure 9

I

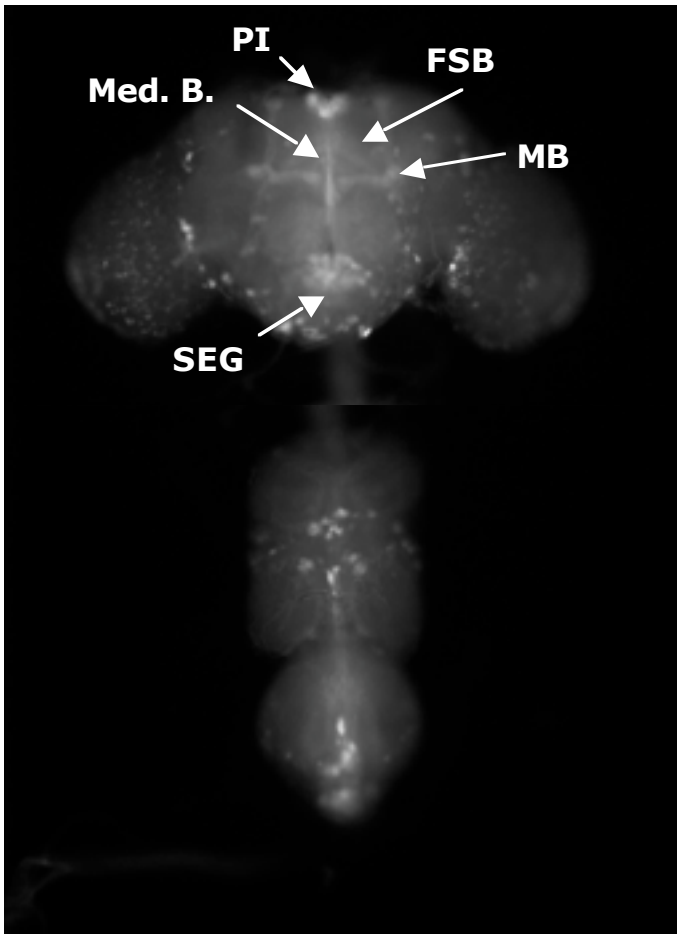


Figure 10

A

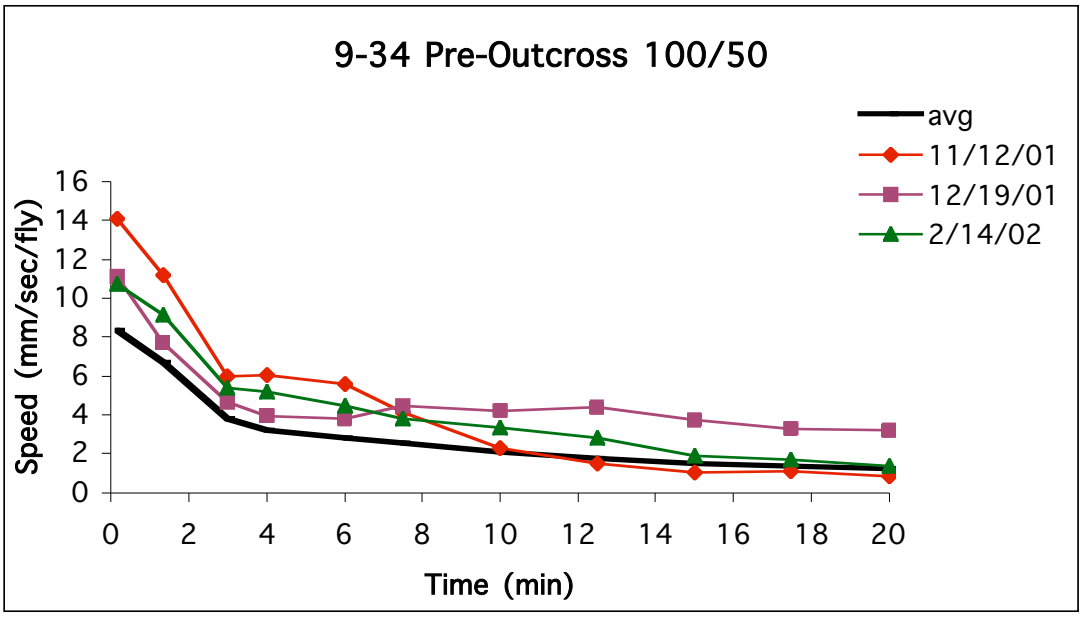
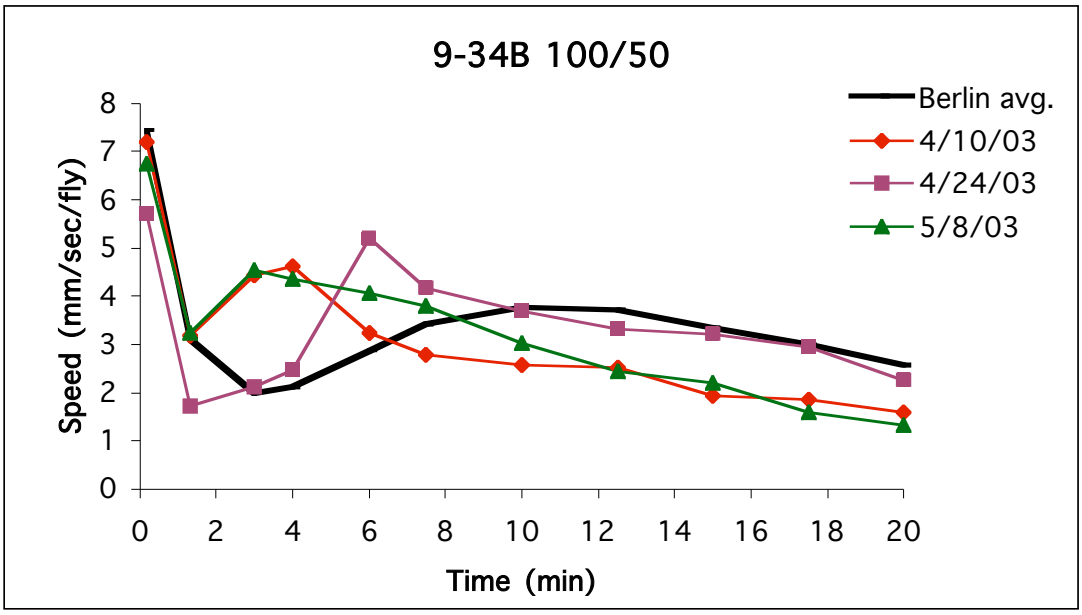

9-34 Pre-Outcross 100/50
avg
11/12/01
12/19/01
2/14/02
Speed (mm/sec/fly)
0
2
4
6
8
10
12
14
16
Time (min)
0
2
4
6
8
10
12
14
16
18
20

Figure 10

B


9-34B 100/50
Berlin avg.
4/10/03
4/24/03
5/8/03
Speed (mm/sec/fly)
Time (min)
0
1
2
3
4
5
6
7
8
0
2
4
6
8
10
12
14
16
18
20

C

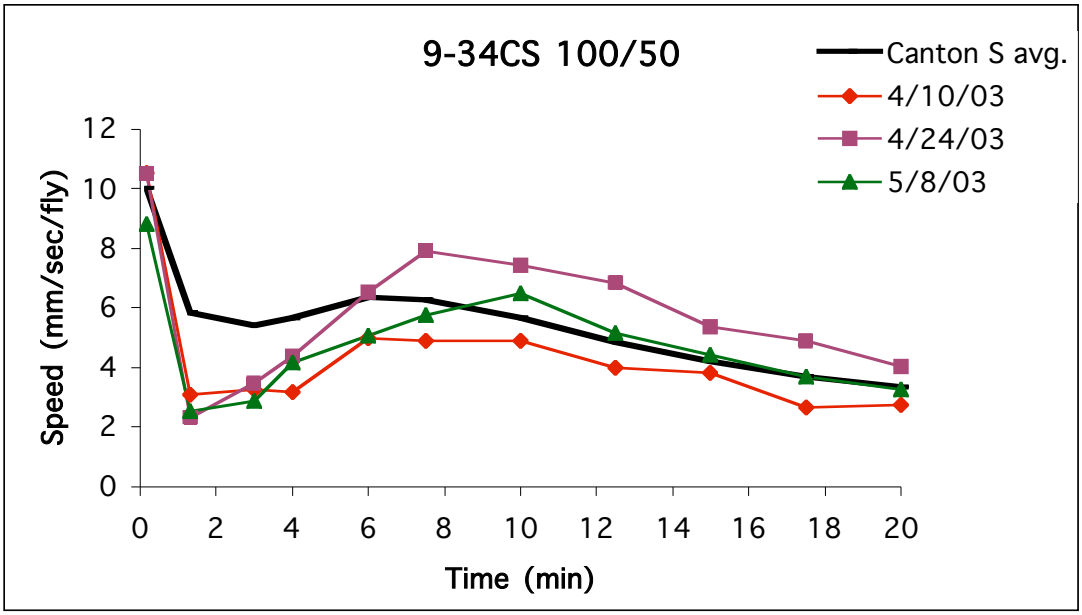
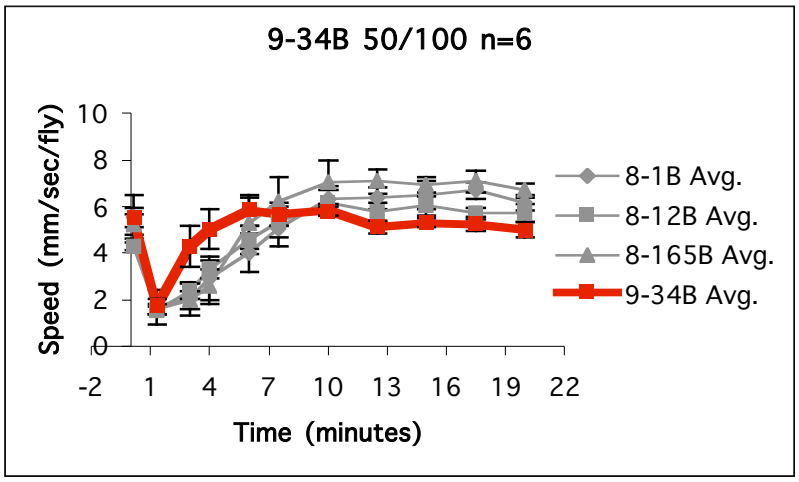
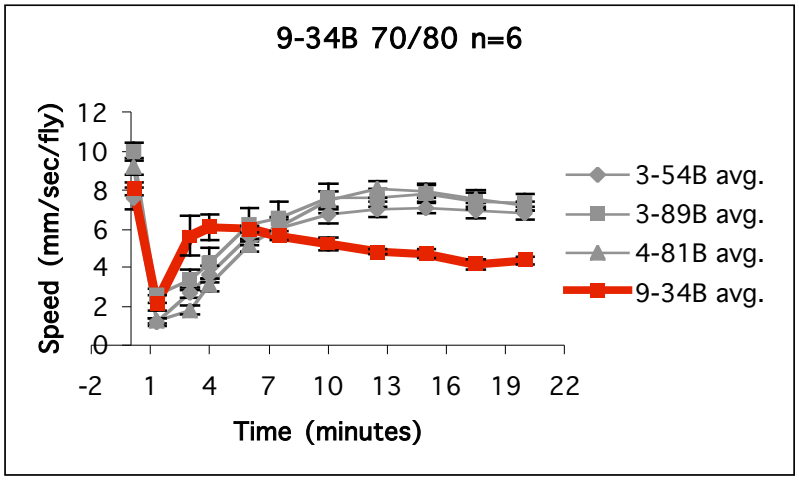

9-34CS 100/50
Canton S avg.
4/10/03
4/24/03
5/8/03
Speed (mm/sec/fly)
Time (min)
0
2
4
6
8
10
12
0
2
4
6
8
10
12
14
16
18
20

Figure 10

D


9-34B 50/100 n=6
Speed (mm/sec/fly)
0
2
4
6
8
10
-2
1
4
7
10
13
16
19
22
Time (minutes)
8-1B Avg.
8-12B Avg.
8-165B Avg.
9-34B Avg.


9-34B 70/80 n=6
Speed (mm/sec/fly)
0
2
4
6
8
10
12
-2
1
4
7
10
13
16
19
22
Time (minutes)
3-54B avg.
3-89B avg.
4-81B avg.
9-34B avg.

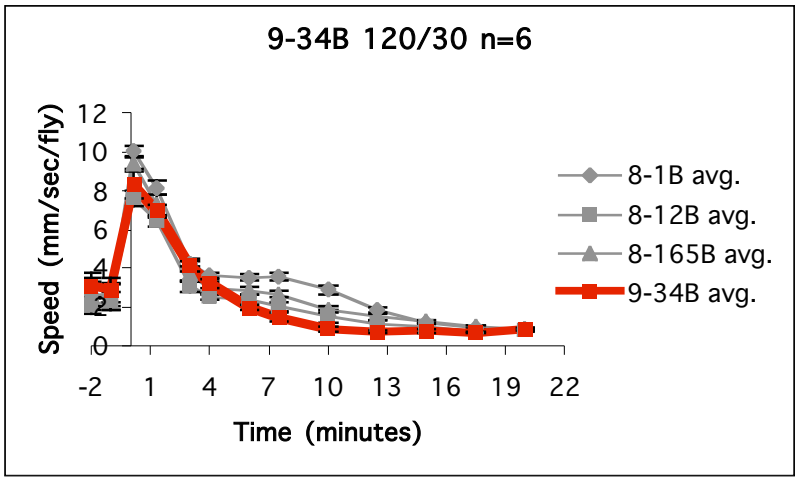
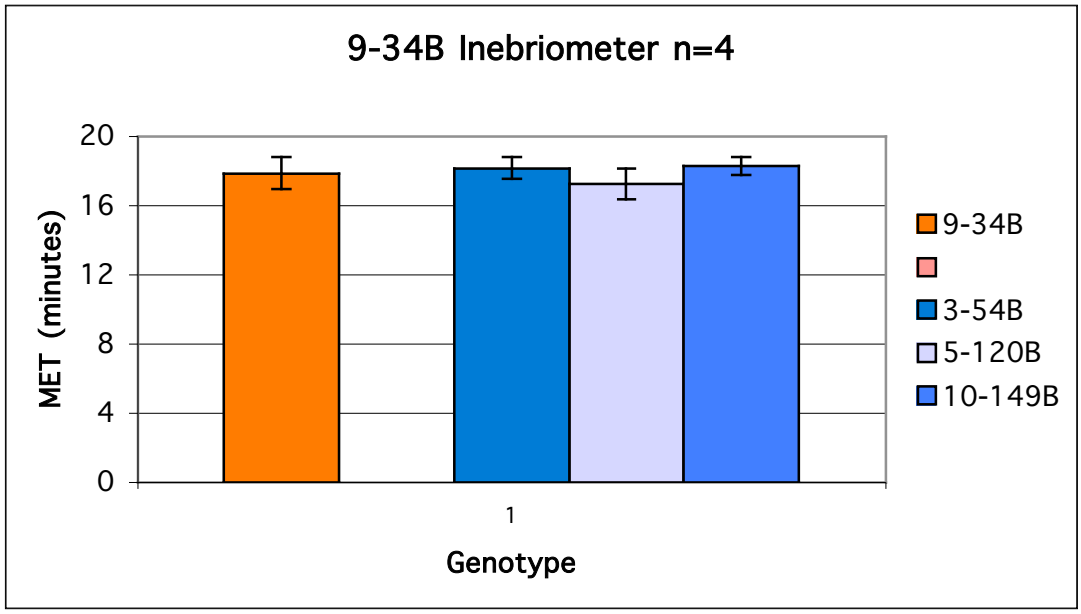

9-34B 120/30 n=6
Speed (mm/sec/fly)
0
2
4
6
8
10
12
-2
1
4
7
10
13
16
19
22
Time (minutes)
8-1B avg.
8-12B avg.
8-165B avg.
9-34B avg.

Figure 10

E


9-34B Inebriometer n=4
MET (minutes)
20
16
12
8
4
0
1
Genotype
9-34B
3-54B
5-120B
10-149B

F

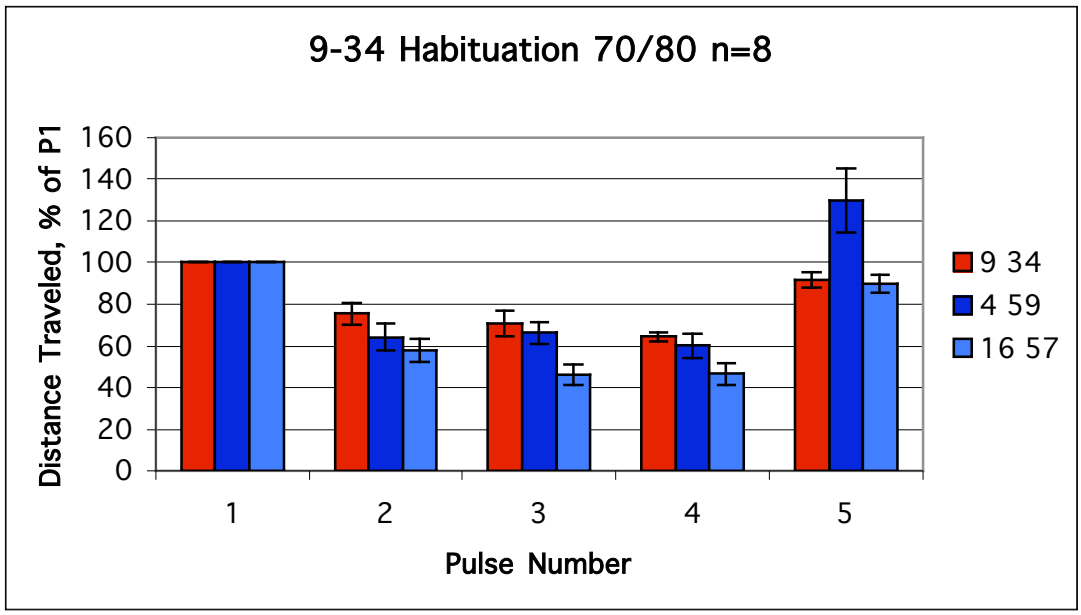
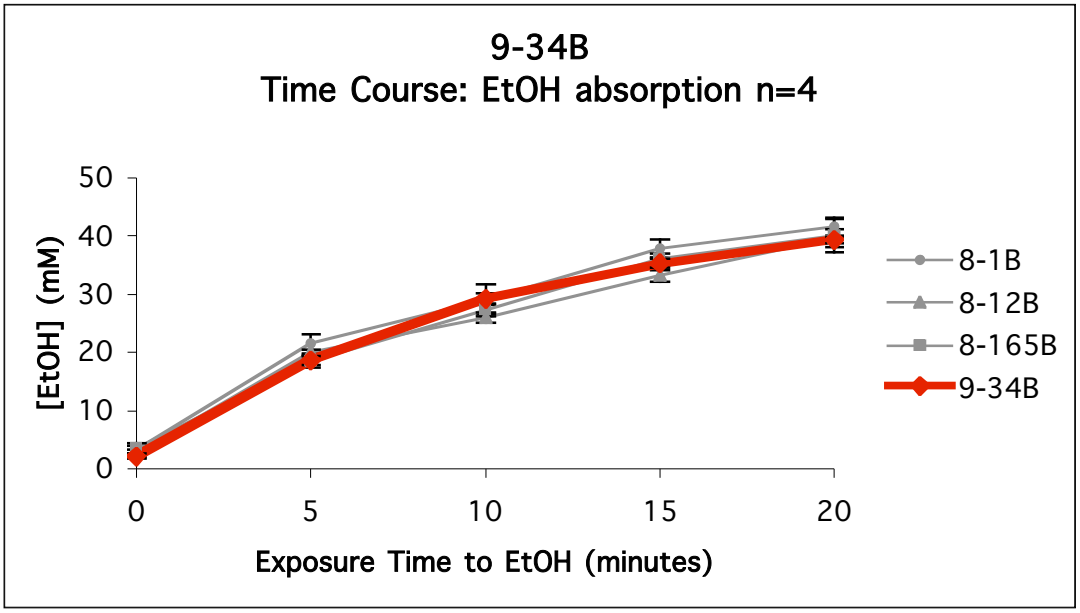

9-34 Habituation 70/80 n=8
Distance Traveled, % of P1
160
140
120
100
80
60
40
20
0
1
2
3
4
5
Pulse Number
9 34
4 59
16 57

Figure 10

G


9-34B
Time Course: EtOH absorption n=4
[EtOH] (mM)
50
40
30
20
10
0
0
5
10
15
20
Exposure Time to EtOH (minutes)
8-1B
8-12B
8-165B
9-34B

H

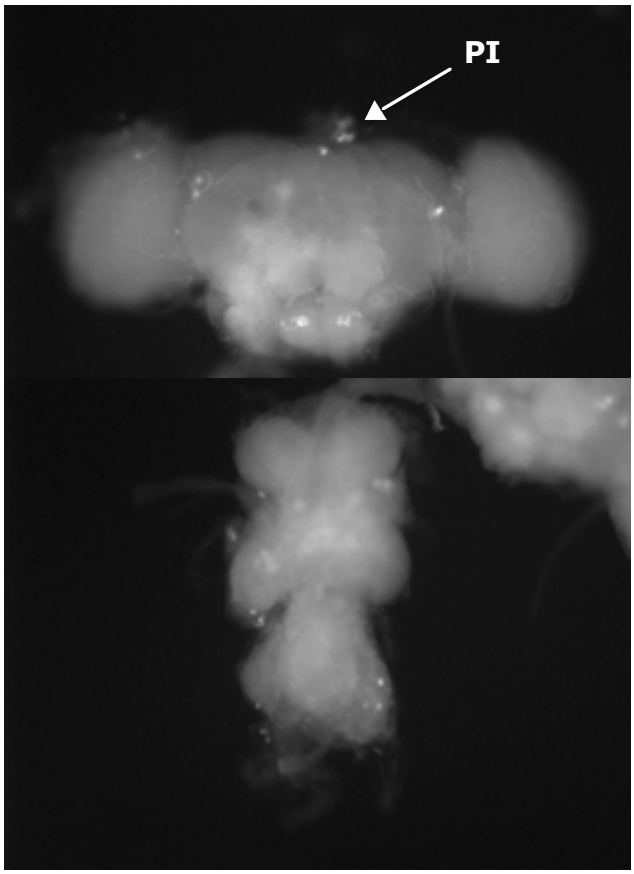

PI

Figure 11

A

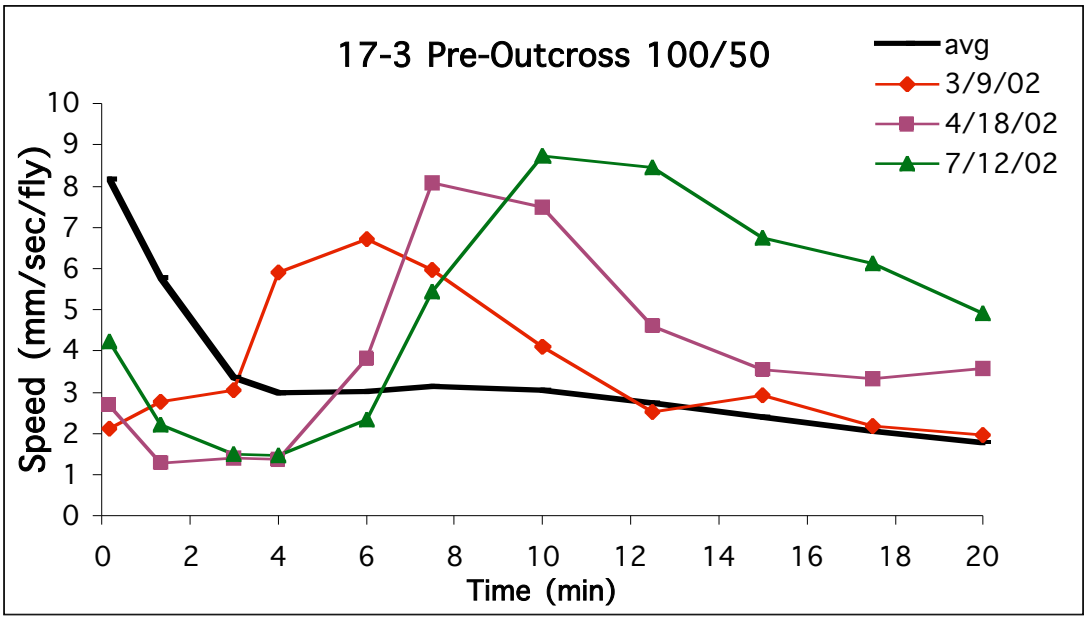
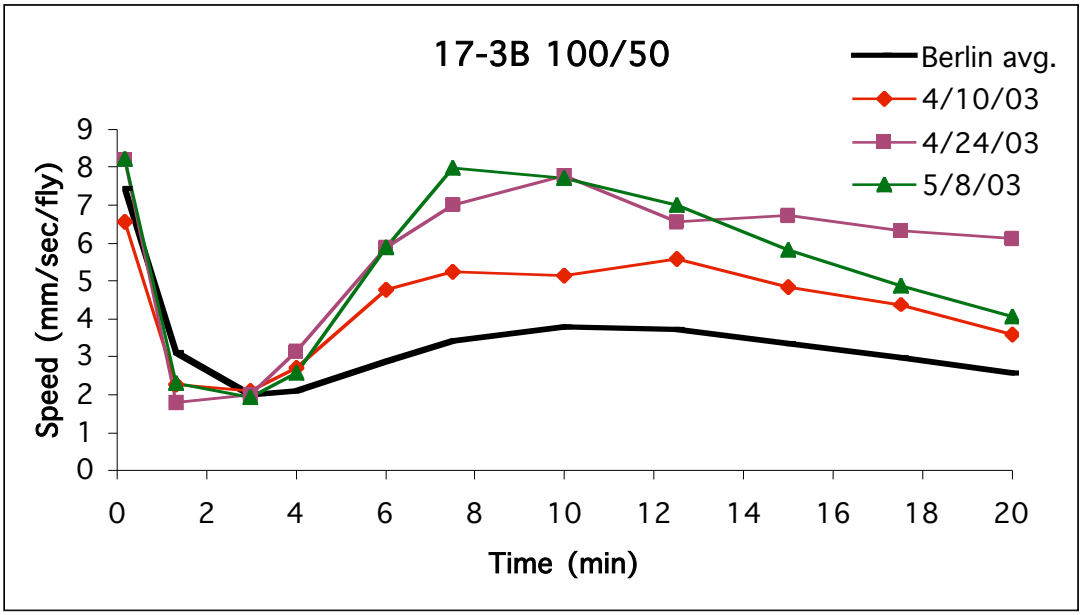

17-3 Pre-Outcross 100/50
avg
3/9/02
4/18/02
7/12/02
Speed (mm/sec/fly)
Time (min)
0
1
2
3
4
5
6
7
8
9
10
0
2
4
6
8
10
12
14
16
18
20

Figure 11

B


17-3B 100/50
Berlin avg.
4/10/03
4/24/03
5/8/03
Speed (mm/sec/fly)
Time (min)
0
1
2
3
4
5
6
7
8
9
0
2
4
6
8
10
12
14
16
18
20

C

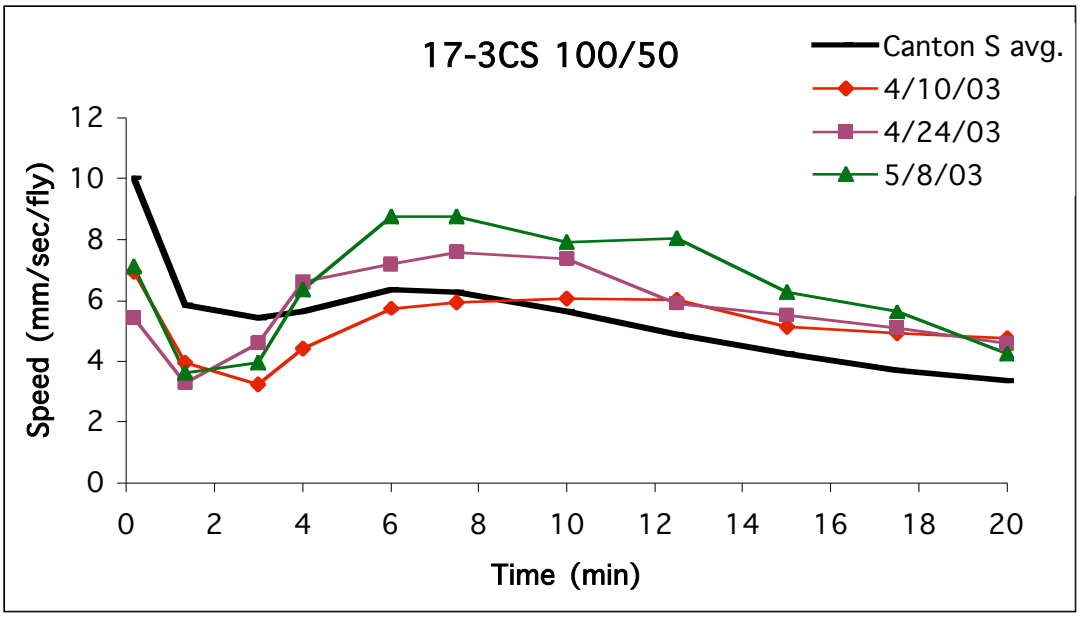

17-3CS 100/50
Canton S avg.
4/10/03
4/24/03
5/8/03
Speed (mm/sec/fly)
Time (min)
0
2
4
6
8
10
12
0
2
4
6
8
10
12
14
16
18
20

Figure 11

D

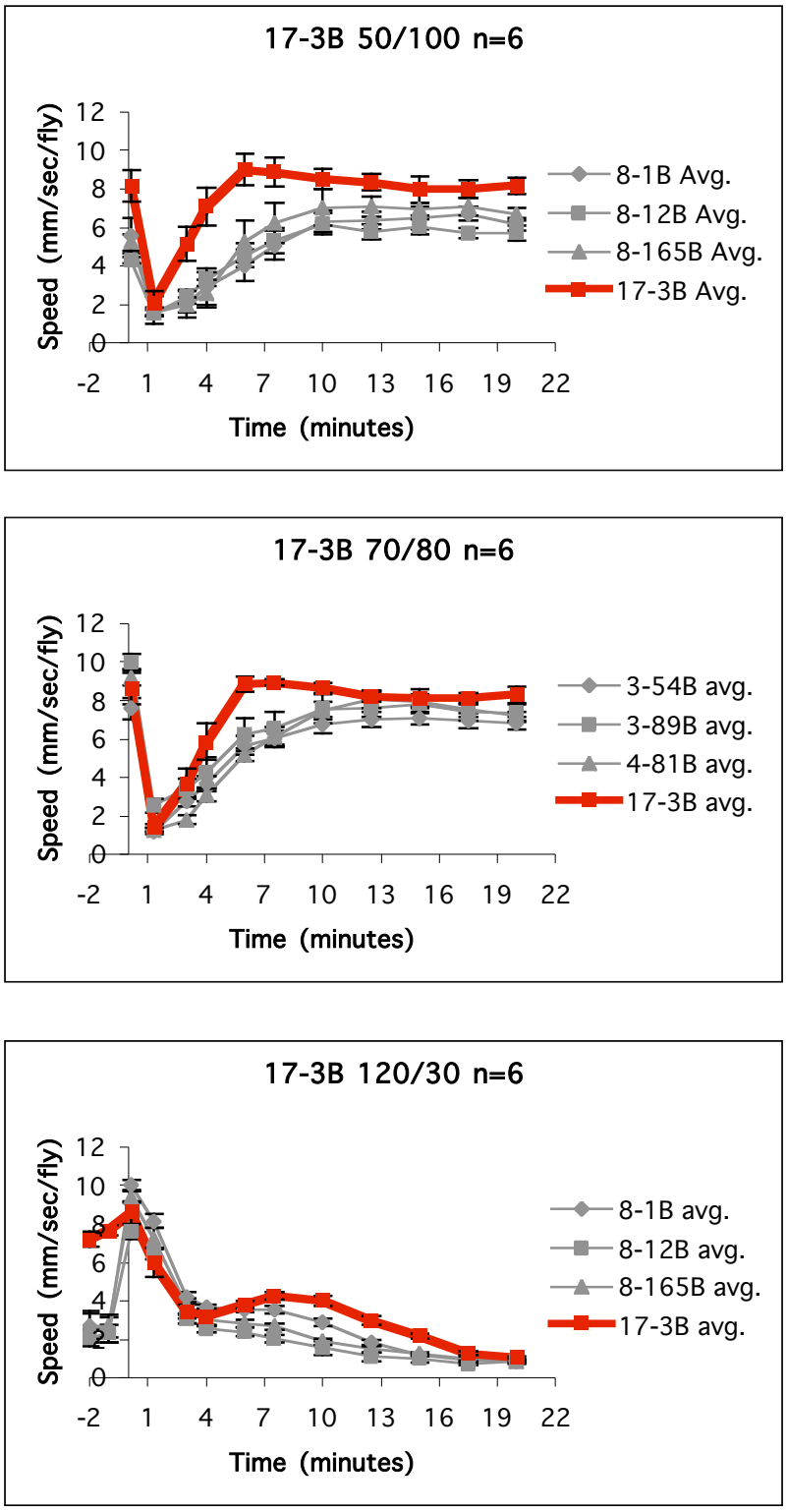
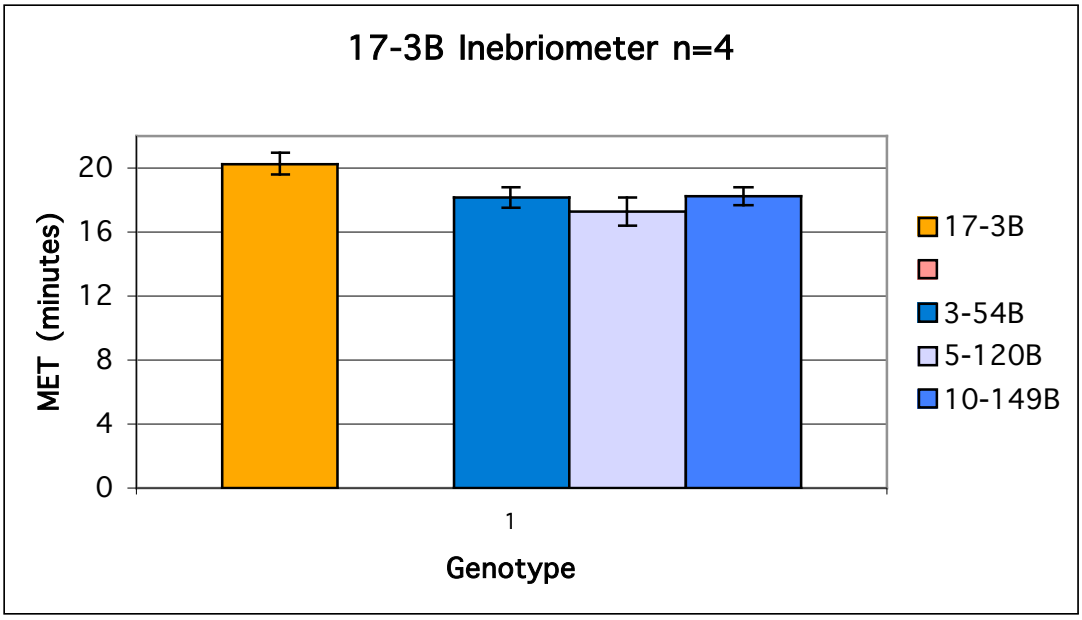

17-3B 50/100 n=6
Speed (mm/sec/fly)
Time (minutes)
8-1B Avg.
8-12B Avg.
8-165B Avg.
17-3B Avg.
17-3B 70/80 n=6
Speed (mm/sec/fly)
Time (minutes)
3-54B avg.
3-89B avg.
4-81B avg.
17-3B avg.
17-3B 120/30 n=6
Speed (mm/sec/fly)
Time (minutes)
8-1B avg.
8-12B avg.
8-165B avg.
17-3B avg.

Figure 11

E


17-3B Inebriometer n=4
MET (minutes)
20
16
12
8
4
0
1
Genotype
17-3B
3-54B
5-120B
10-149B

F

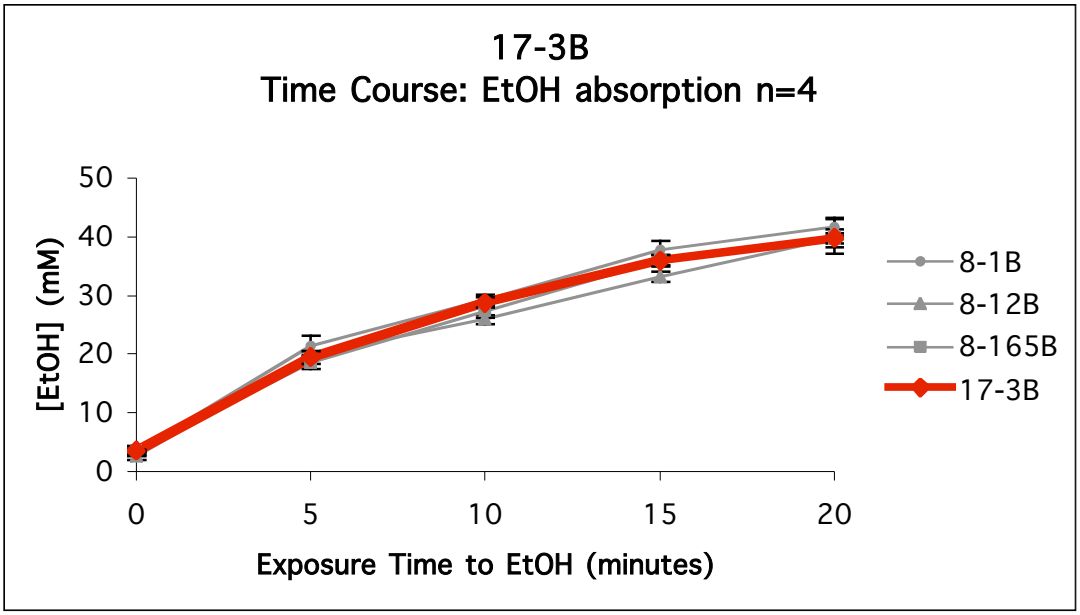

17-3B
Time Course: EtOH absorption n=4
[EtOH] (mM)
50
40
30
20
10
0
0
5
10
15
20
Exposure Time to EtOH (minutes)
8-1B
8-12B
8-165B
17-3B

Figure 11

G

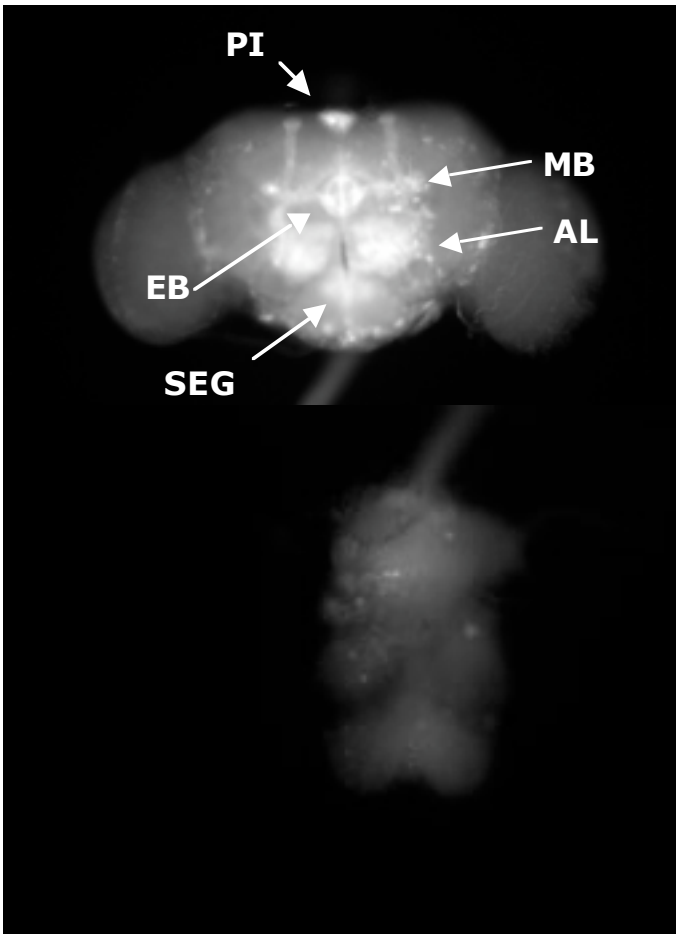


Figure 12

A

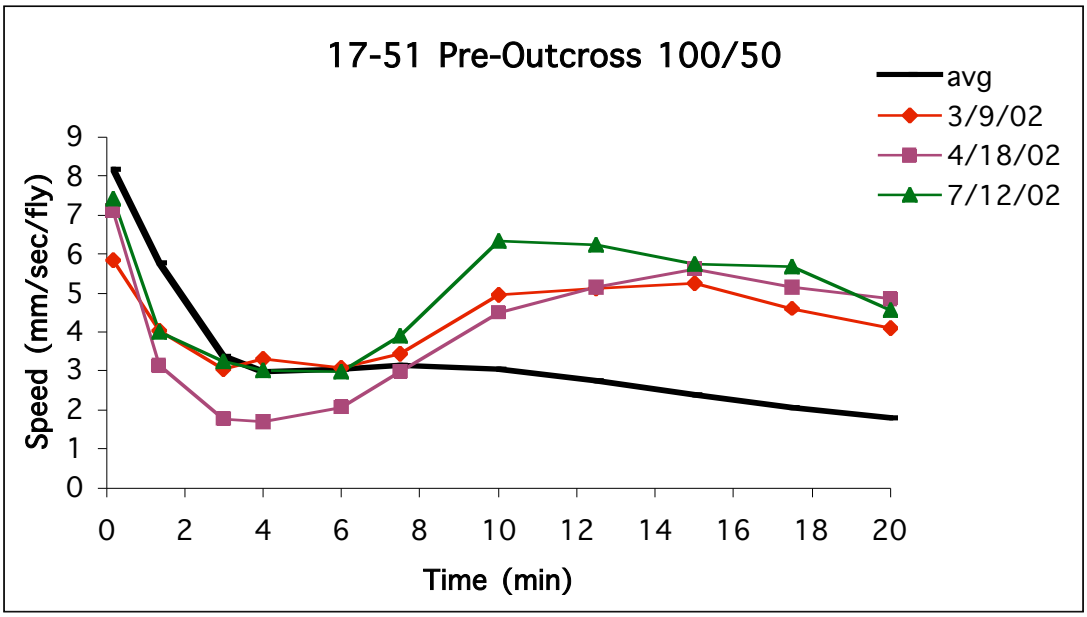
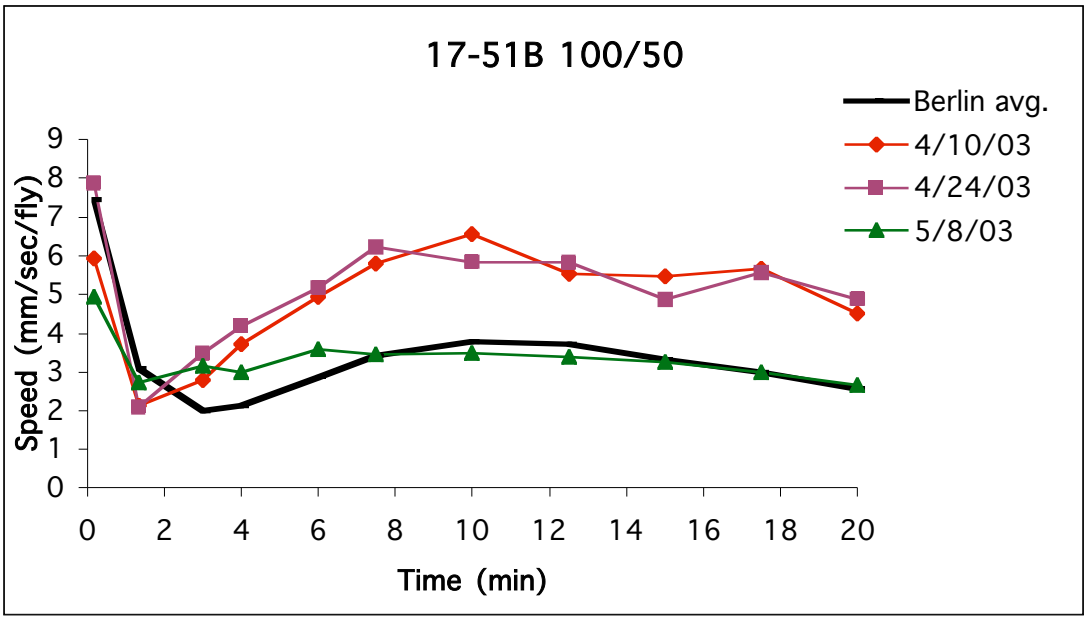

17-51 Pre-Outcross 100/50
avg
3/9/02
4/18/02
7/12/02
Speed (mm/sec/fly)
0
1
2
3
4
5
6
7
8
9
0
2
4
6
8
10
12
14
16
18
20
Time (min)

Figure 12

B


17-51B 100/50
Berlin avg.
4/10/03
4/24/03
5/8/03
Speed (mm/sec/fly)
Time (min)
0
1
2
3
4
5
6
7
8
9
0
2
4
6
8
10
12
14
16
18
20

C

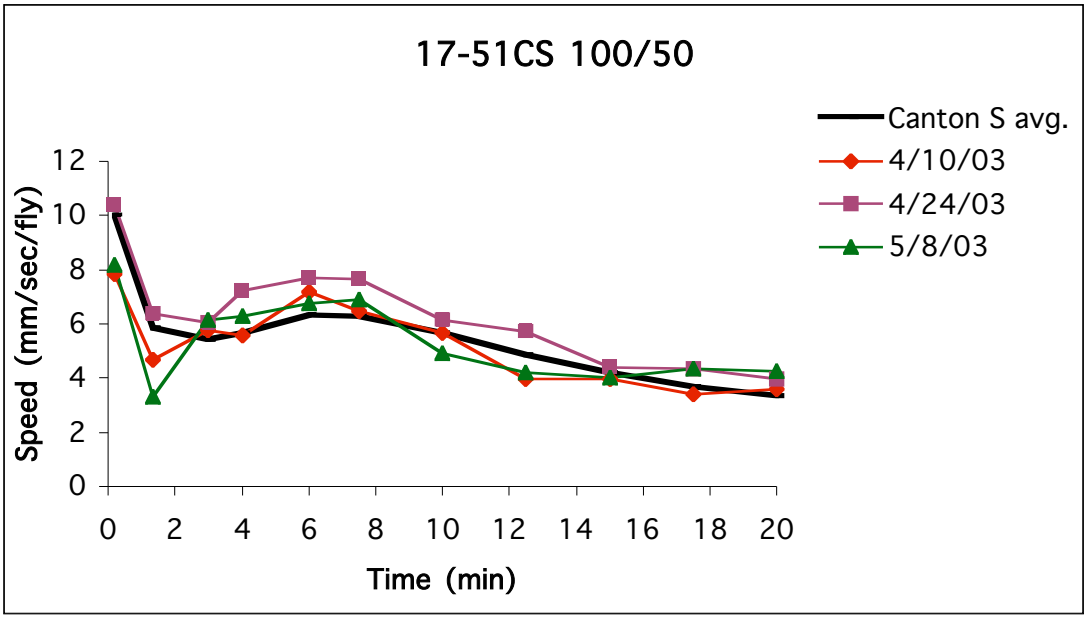
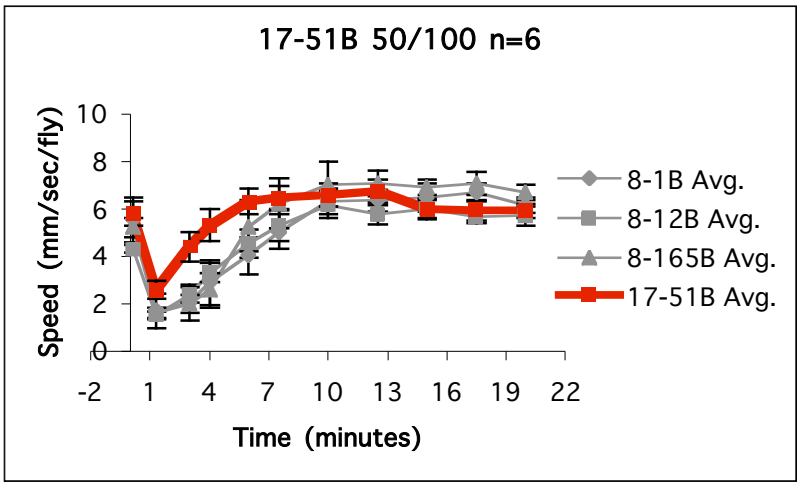
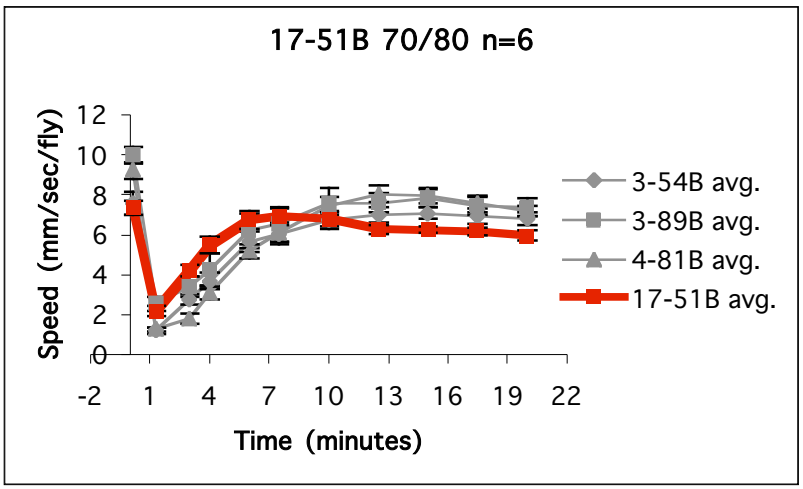

17-51CS 100/50
Canton S avg.
4/10/03
4/24/03
5/8/03
Speed (mm/sec/fly)
Time (min)
0
2
4
6
8
10
12
0
2
4
6
8
10
12
14
16
18
20

Figure 12

D


17-51B 50/100 n=6
Speed (mm/sec/fly)
0
2
4
6
8
10
-2
1
4
7
10
13
16
19
22
Time (minutes)
8-1B Avg.
8-12B Avg.
8-165B Avg.
17-51B Avg.


17-51B 70/80 n=6
Speed (mm/sec/fly)
0
2
4
6
8
10
12
-2
1
4
7
10
13
16
19
22
Time (minutes)
3-54B avg.
3-89B avg.
4-81B avg.
17-51B avg.

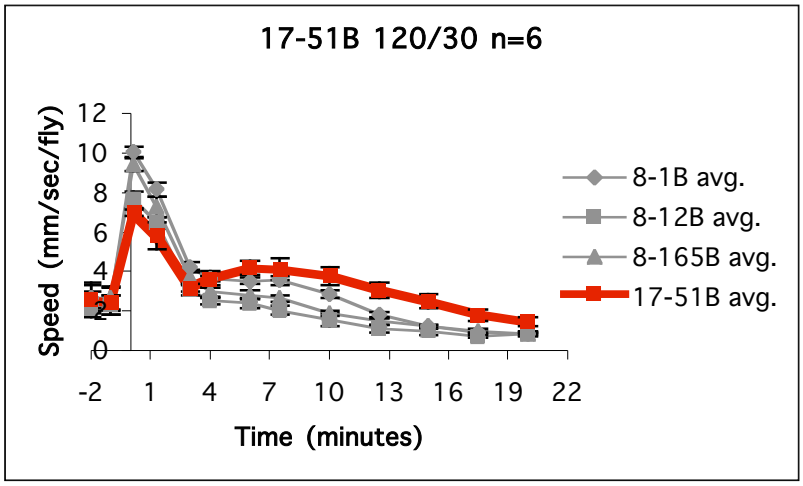
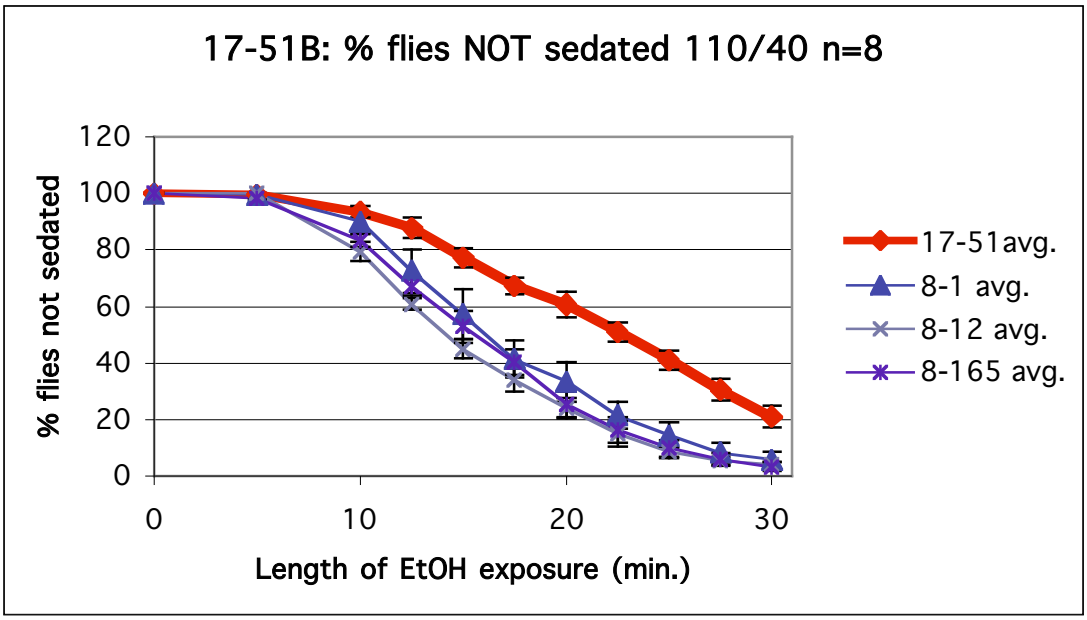

17-51B 120/30 n=6
Speed (mm/sec/fly)
0
2
4
6
8
10
12
-2
1
4
7
10
13
16
19
22
Time (minutes)
8-1B avg.
8-12B avg.
8-165B avg.
17-51B avg.

Figure 12

E


17-51B: % flies NOT sedated 110/40 n=8
% flies not sedated
120
100
80
60
40
20
0
0
10
20
30
Length of EtOH exposure (min.)
17-51avg.
8-1 avg.
8-12 avg.
8-165 avg.

F

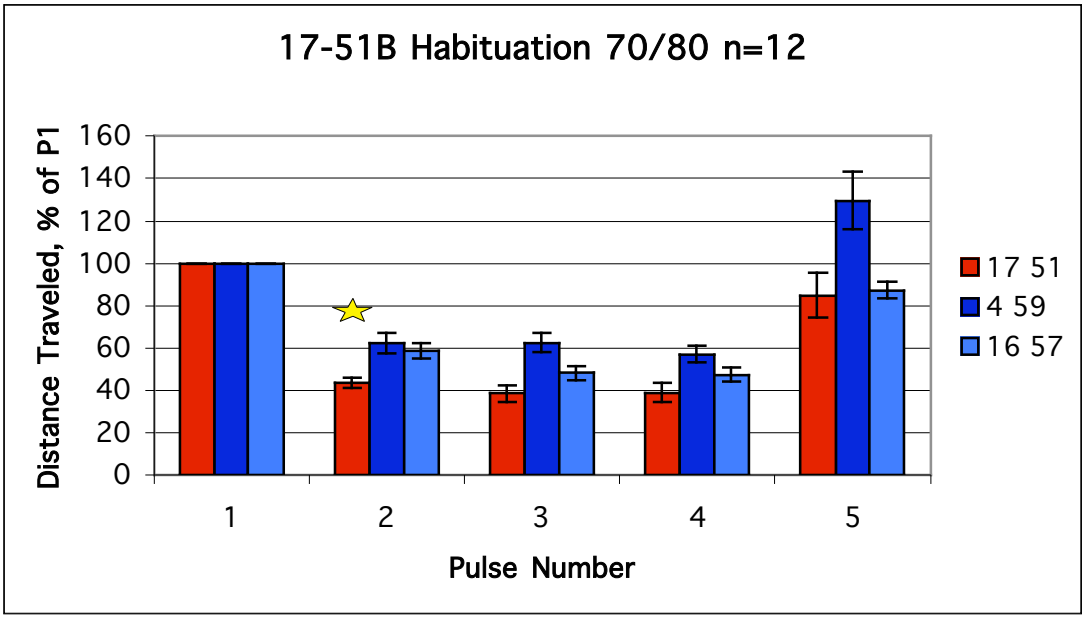
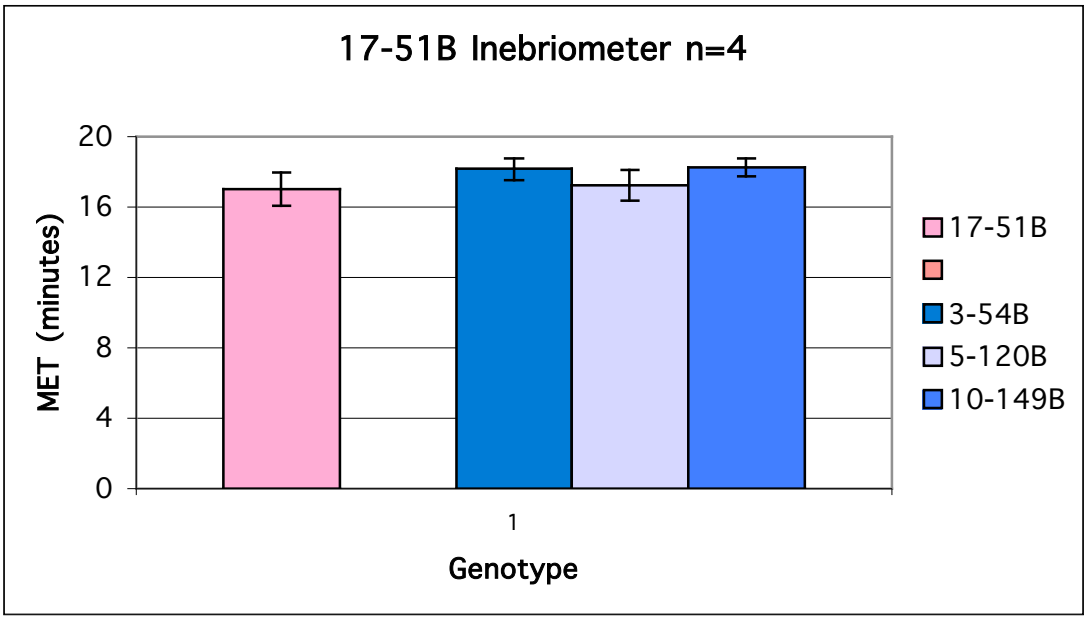

17-51B Habituation 70/80 n=12
Distance Traveled, % of P1
160
140
120
100
80
60
40
20
0
1
2
3
4
5
Pulse Number
17 51
4 59
16 57

Figure 12

G


17-51B Inebriometer n=4
20
16
12
8
4
0
MET (minutes)
1
Genotype
17-51B
3-54B
5-120B
10-149B

H

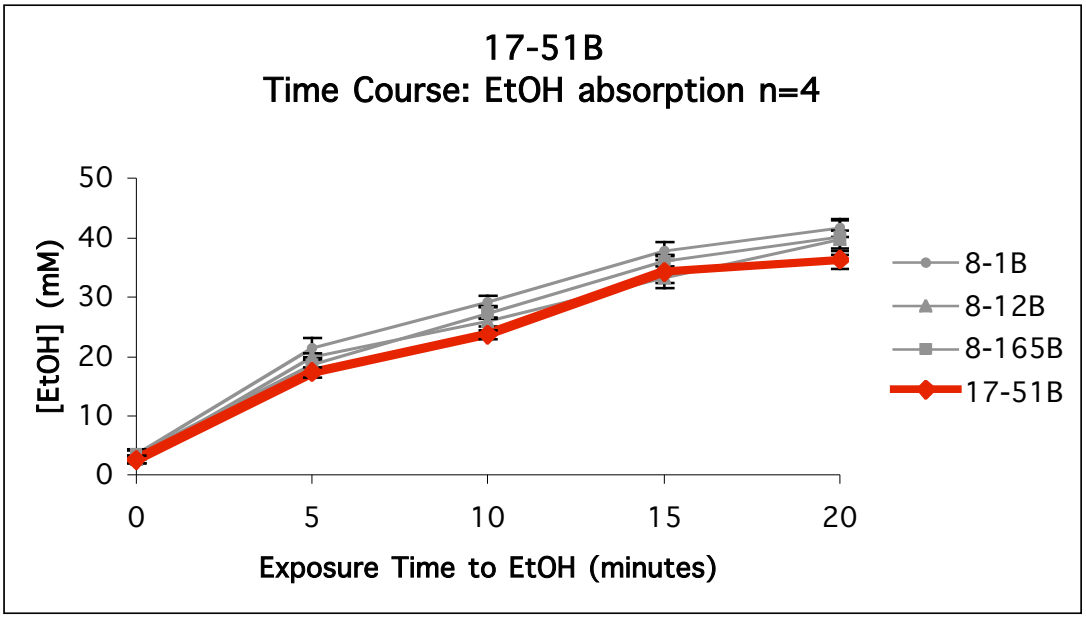

17-51B
Time Course: EtOH absorption n=4
50
40
30
20
10
0
[EtOH] (mM)
0
5
10
15
20
Exposure Time to EtOH (minutes)
8-1B
8-12B
8-165B
17-51B

Figure 12

I

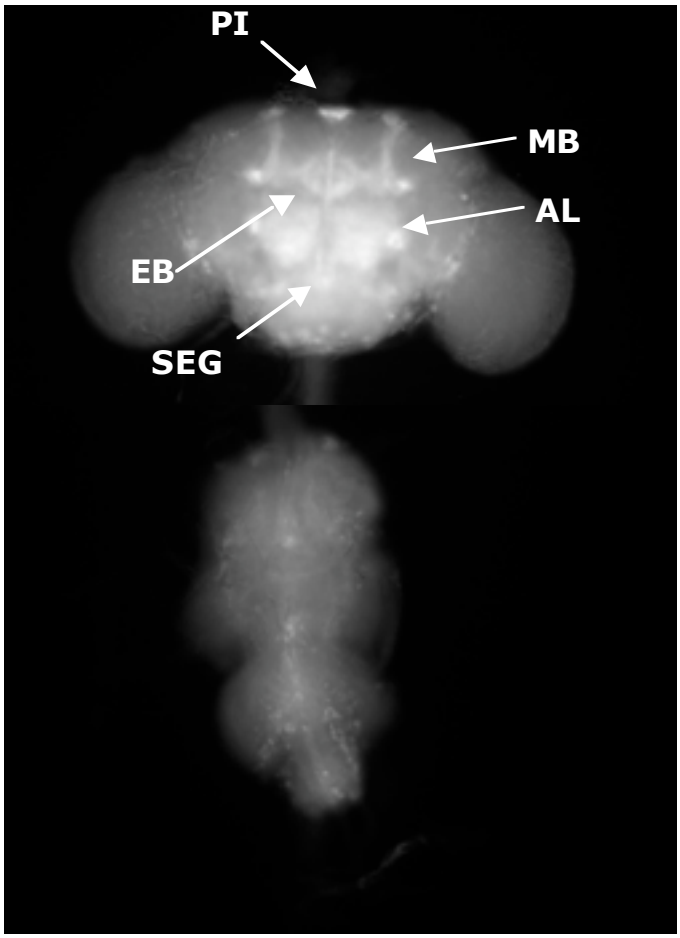
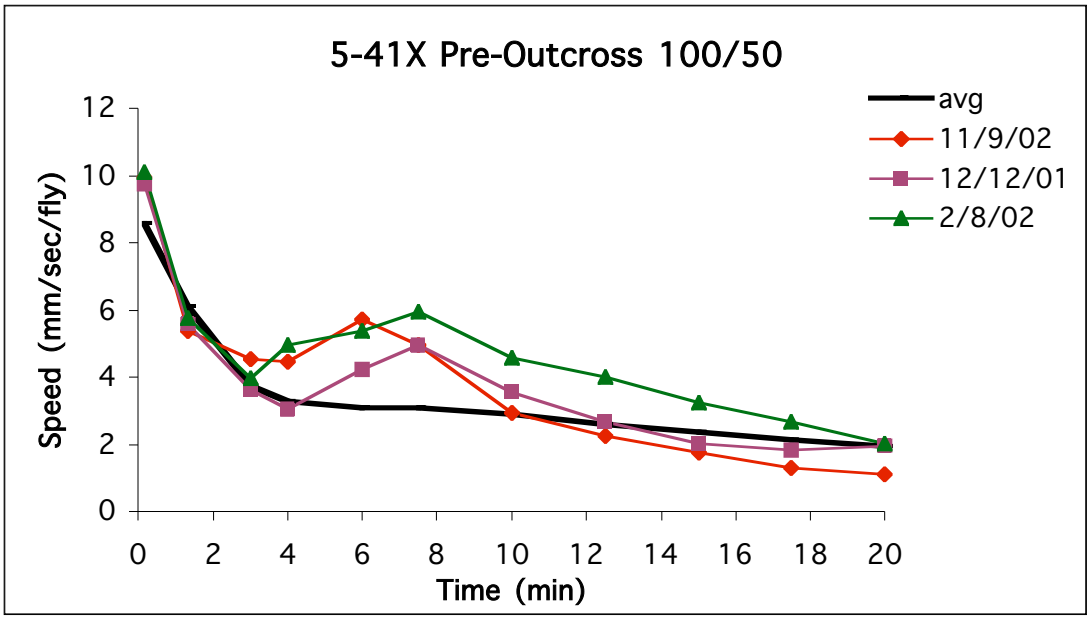


Figure 13

A


5-41X Pre-Outcross 100/50
avg
11/9/02
12/12/01
2/8/02
Speed (mm/sec/fly)
Time (min)
0
2
4
6
8
10
12
14
16
18
20

B

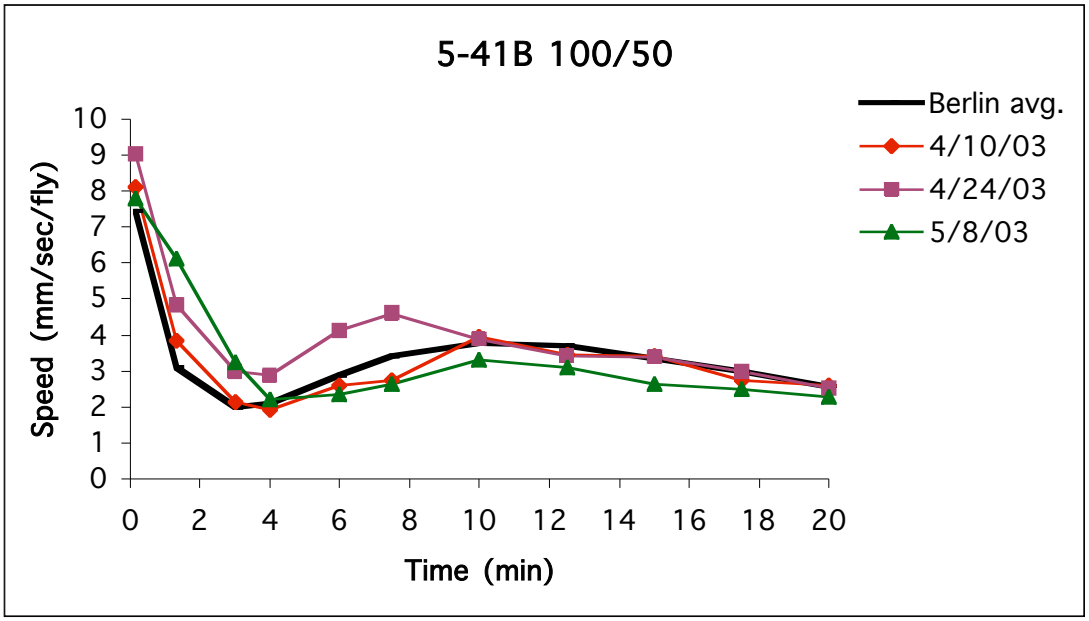
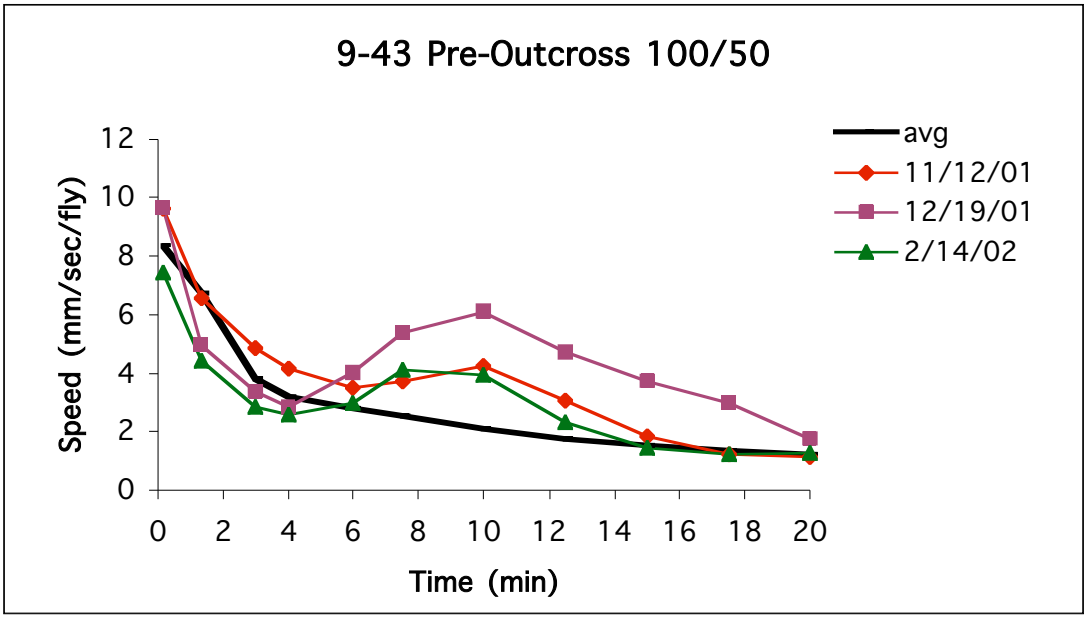

5-41B 100/50
Berlin avg.
4/10/03
4/24/03
5/8/03
Speed (mm/sec/fly)
Time (min)
0
1
2
3
4
5
6
7
8
9
10
12
14
16
18
20

Figure 13

C


9-43 Pre-Outcross 100/50
Speed (mm/sec/fly)
Time (min)
avg
11/12/01
12/19/01
2/14/02

D

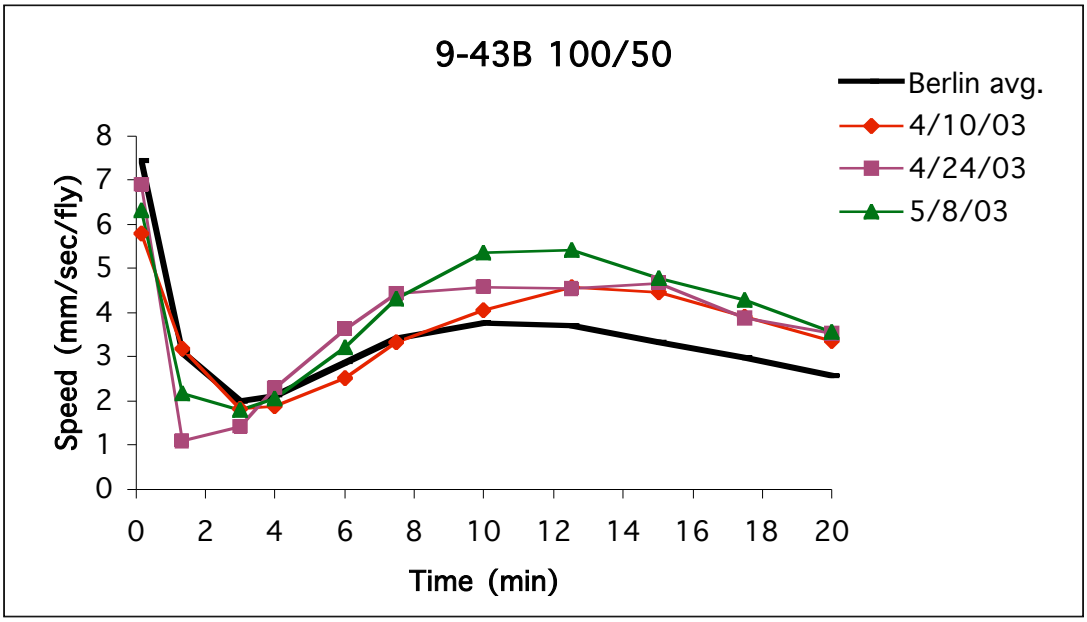
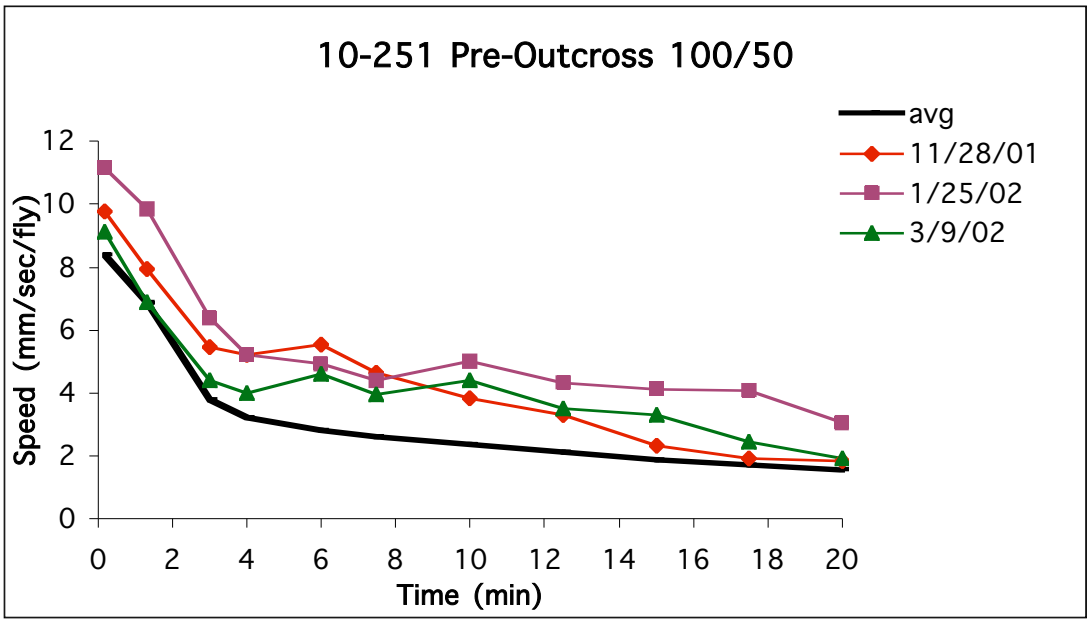

9-43B 100/50
Speed (mm/sec/fly)
Time (min)
Berlin avg.
4/10/03
4/24/03
5/8/03

Figure 13

E


10-251 Pre-Outcross 100/50
avg
11/28/01
1/25/02
3/9/02
Speed (mm/sec/fly)
Time (min)
0
2
4
6
8
10
12
14
16
18
20

F

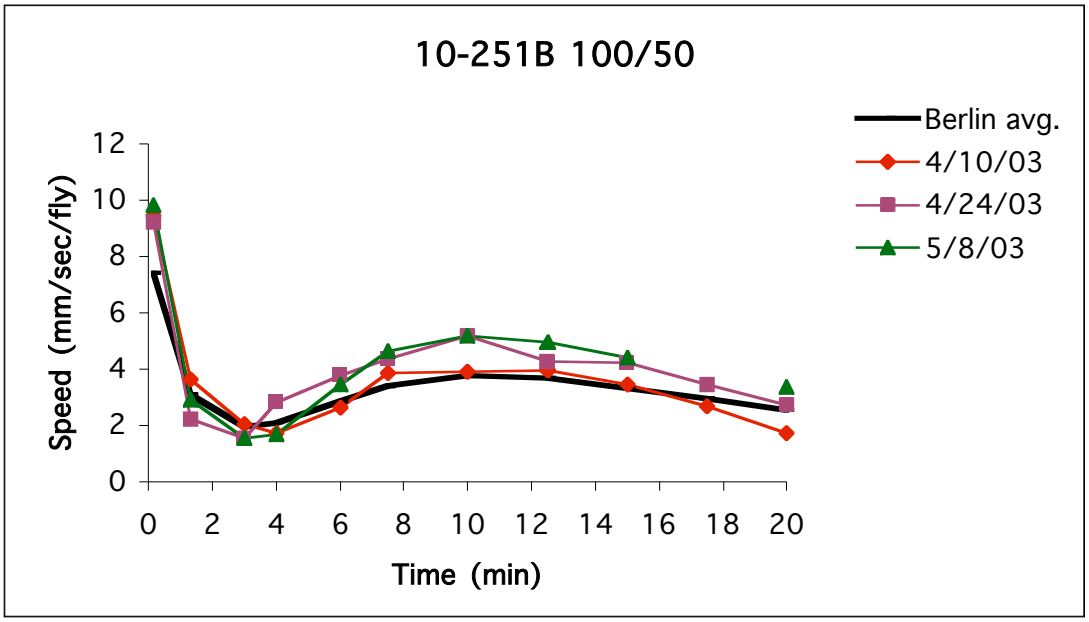
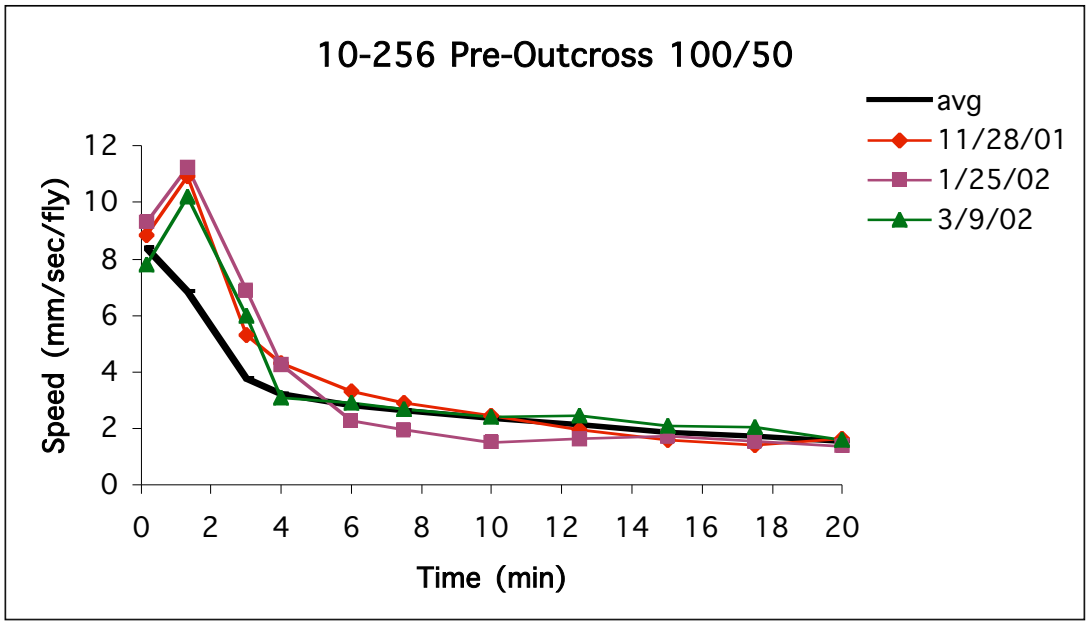

10-251B 100/50
Berlin avg.
4/10/03
4/24/03
5/8/03
Speed (mm/sec/fly)
Time (min)
0
2
4
6
8
10
12
14
16
18
20

Figure 13

G


10-256 Pre-Outcross 100/50
avg
11/28/01
1/25/02
3/9/02
Speed (mm/sec/fly)
Time (min)
0
2
4
6
8
10
12
14
16
18
20

H

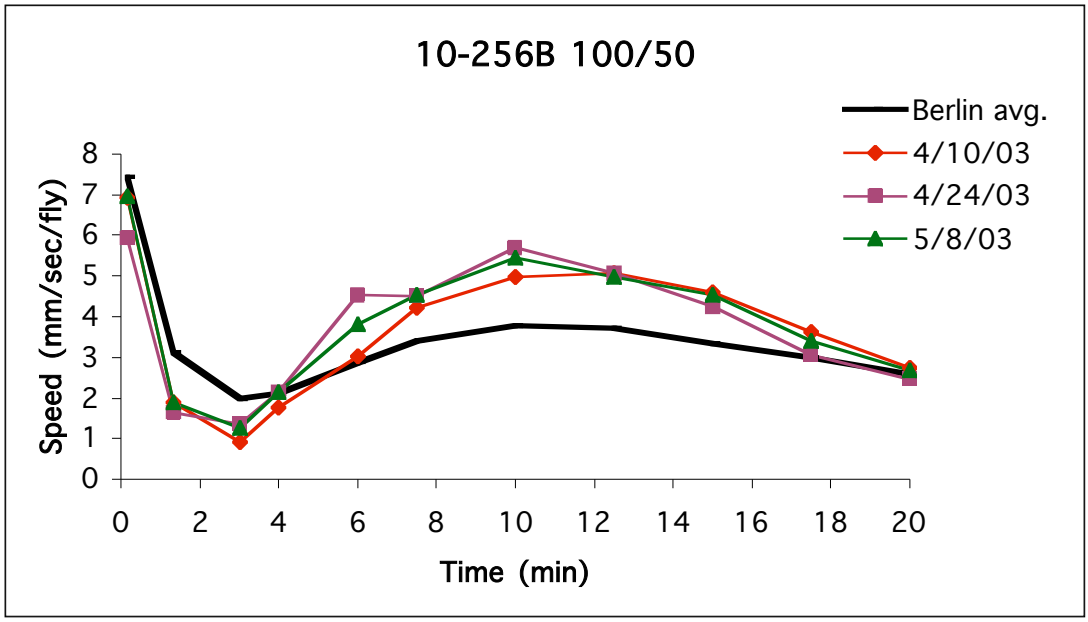
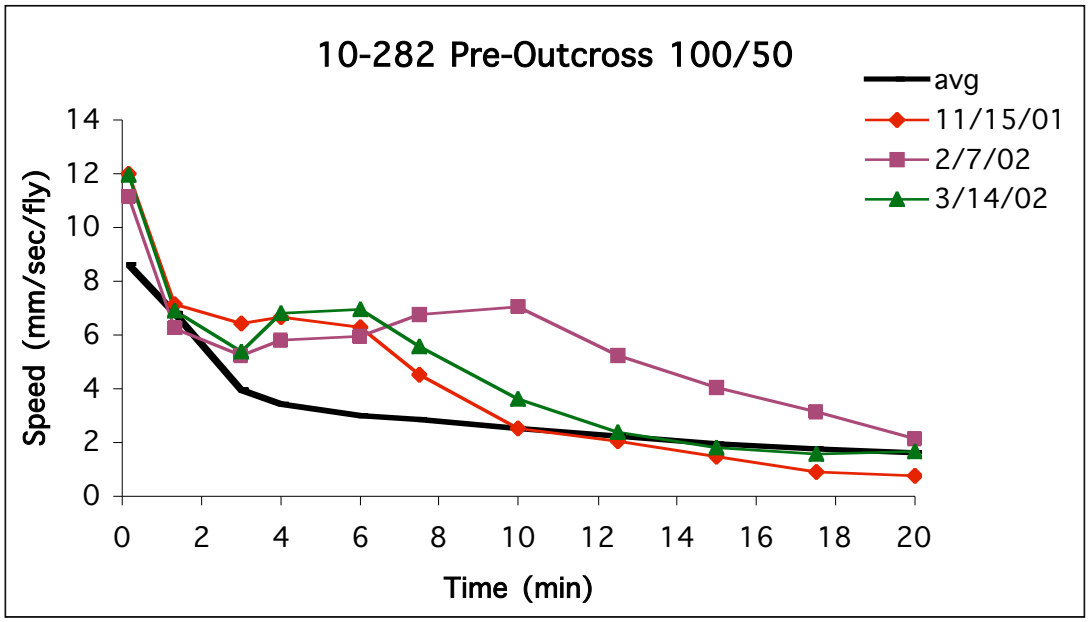

10-256B 100/50
Berlin avg.
4/10/03
4/24/03
5/8/03
Speed (mm/sec/fly)
Time (min)
0
1
2
3
4
5
6
7
8
0
2
4
6
8
10
12
14
16
18
20

Figure 13

I


10-282 Pre-Outcross 100/50
avg
11/15/01
2/7/02
3/14/02
Speed (mm/sec/fly)
Time (min)
0
2
4
6
8
10
12
14
16
18
20

J

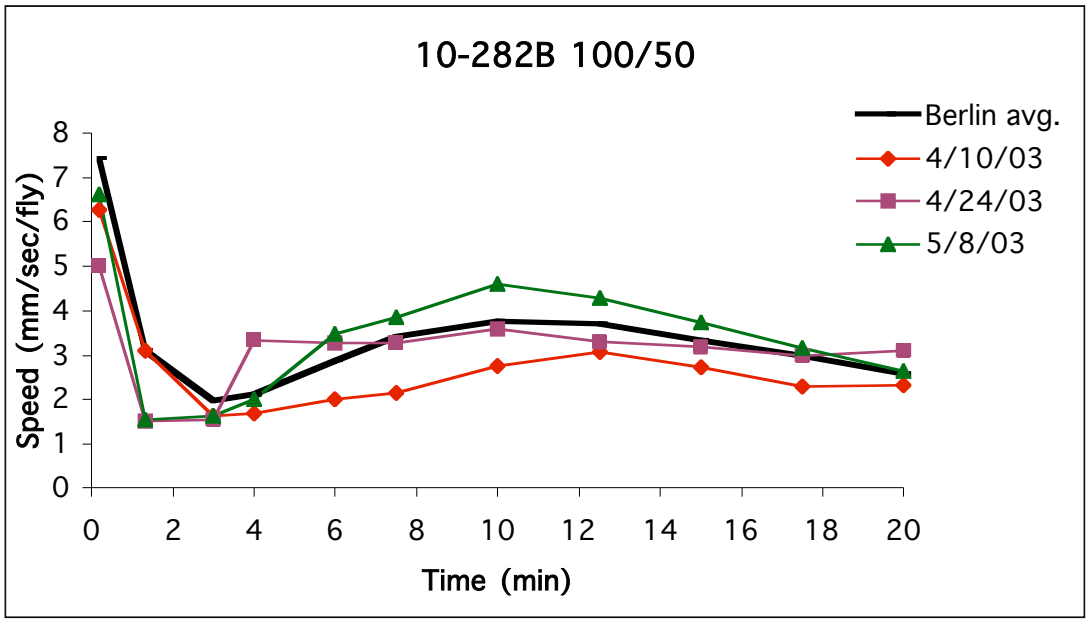
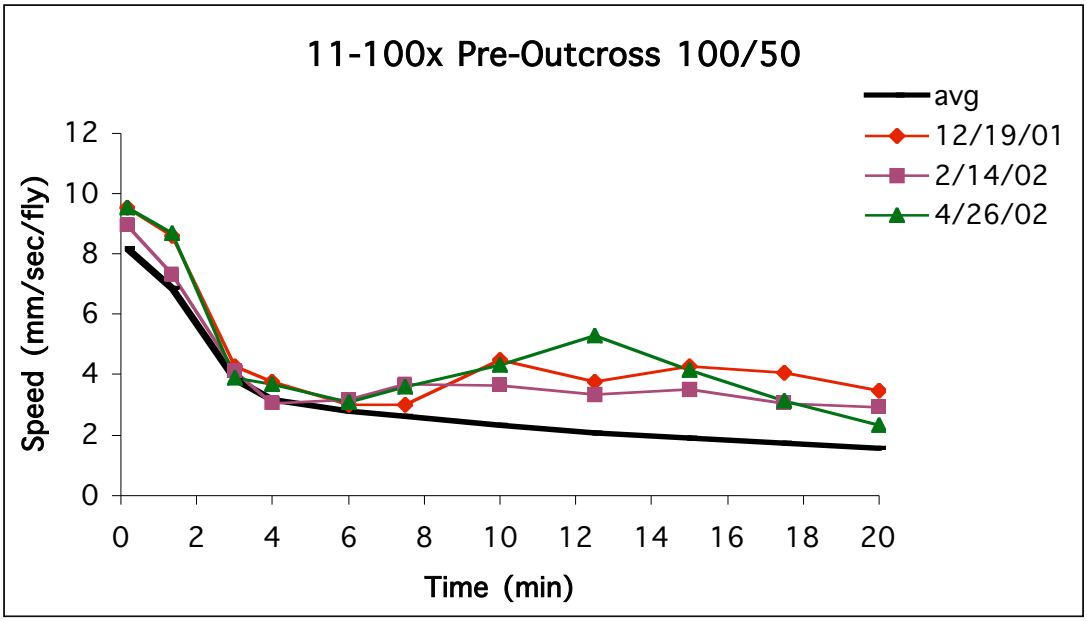
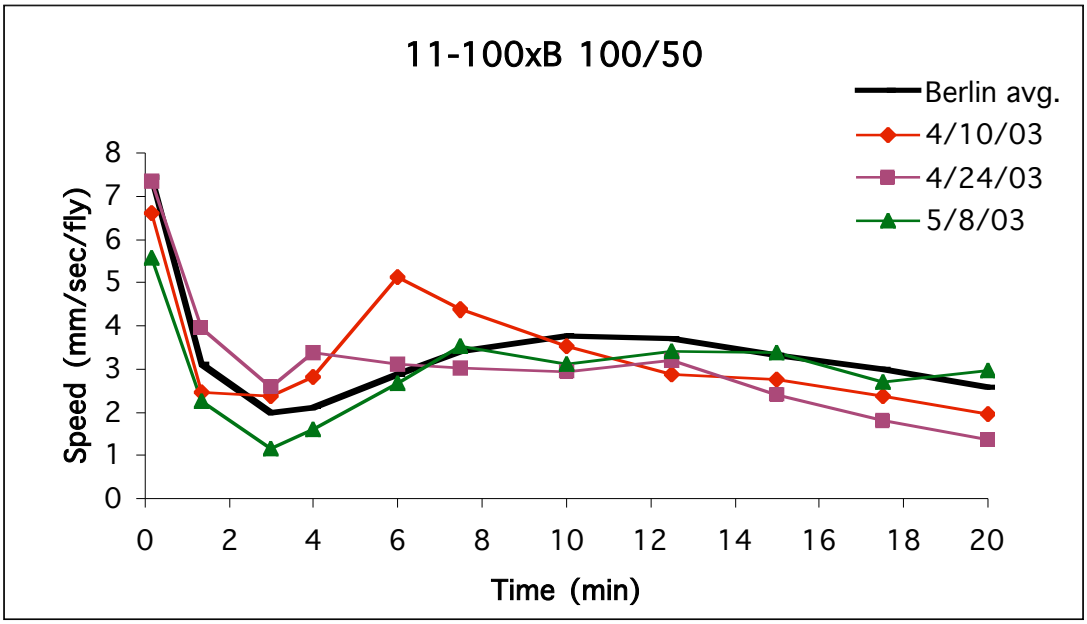

10-282B 100/50
Berlin avg.
4/10/03
4/24/03
5/8/03
Speed (mm/sec/fly)
Time (min)
0
1
2
3
4
5
6
7
8
0
2
4
6
8
10
12
14
16
18
20

Figure 13

K


11-100x Pre-Outcross 100/50
avg
12/19/01
2/14/02
4/26/02
Speed (mm/sec/fly)
Time (min)
0
2
4
6
8
10
12
14
16
18
20

L


11-100xB 100/50
Berlin avg.
4/10/03
4/24/03
5/8/03
Speed (mm/sec/fly)
Time (min)
0
1
2
3
4
5
6
7
8
0
2
4
6
8
10
12
14
16
18
20

Chapter 3

Happyhour, a Ste20 Family Kinase that Implicates Neuronal EGFR Signaling in Ethanol-Induced Sedation in *Drosophila*

Ammon B. Corl, Karen H. Berger, Julie D. Gesch, and Ulrike Heberlein

Abstract

In an effort to expand our knowledge of the genetic components underlying the behavioral effects of ethanol, we conducted a genetic screen in *Drosophila* and identified a mutant, *happyhour* (*hppy*), that is resistant to the sedative effects of ethanol. While loss of function mutations in *hppy* resulted in resistance to ethanol-induced sedation, neuronal overexpression of *hppy* caused increased sensitivity. Although *hppy* shows strong homology to mammalian Ste20 family kinases involved in JNK signaling, we found that neither activation nor inhibition of the JNK pathway affected ethanol sedation. Interestingly, perturbations of a second MAP kinase pathway, the EGF receptor (EGFR)/ERK pathway, in neuronal tissues strongly affected sensitivity to ethanol-induced sedation. Genetic interaction experiments between *hppy* and the EGFR/ERK pathway suggest a role for *hppy* as an inhibitor of the pathway, functioning downstream of the EGFR but upstream of ERK. Our data identifies *hppy* as a novel modulator of EGFR/ERK signaling and uncovers a previously uncharacterized role for this pathway in mediating the behavioral response to ethanol in *Drosophila*.

Introduction

Alcohol (ethanol) is one of the most popularly consumed and abused drugs in the world. The pleasurable and disinhibiting effects of alcohol consumption have been enjoyed by humankind for thousands of years. For some, however, alcohol consumption leads to alcohol addiction, a devastating illness with enormous medical and societal costs. In the United States, for example, approximately 7% of adults are alcoholics, and alcohol-related problems cost the country over $175 billion per year, and are responsible

for 100,000 deaths (Diamond and Gordon, 1997; Volpicelli, 2001). A better understanding of the genetic and environmental factors that contribute to the development of alcoholism would provide considerable benefits to those who suffer from alcohol addiction and to society in general.

Although the cognitive and behavioral changes associated with alcohol consumption are quite familiar to many of us, our knowledge concerning the mechanisms through which ethanol acts on the central nervous system to produce these behavioral changes is still far from complete. Rather than acting on a single molecular target, *in vitro* studies have shown that ethanol exerts effects on multiple different voltage-gated and ligand-gated ion channels. For example, ethanol has been found to potentiate the function of γ aminobutyric acid-A ($GABA_A$) receptors and inhibit N-methyl-D-aspartate (NMDA) receptors at pharmacologically relevant ethanol concentrations (Diamond and Gordon, 1997; Lovinger, 1997). More recently, studies of genetically engineered mice have provided further insight into some molecules that regulate the behavioral response to ethanol *in vivo*, demonstrating roles for serotonin, dopamine, and cannabinoid systems, as well as several signal transduction pathways (Crabbe et al., 2006).

Family, adoption, and twin studies strongly show a genetic component to alcoholism, although thus far attempts in humans to identify specific genes underlying alcoholism have been largely unsuccessful (Reich et al., 1999; Schuckit et al., 2004). Interestingly, human studies also indicate that the level of response to intoxicating doses of ethanol act as a predictor of future alcoholism (Schuckit et al., 2004). For example, a low level of response to ethanol at age 20 was found to be associated with a four-fold increased likelihood of development of alcoholism within the following ten years

(Schuckit, 1994). This strongly suggests that the identification of genes and pathways mediating acute responses to ethanol promises to offer helpful insight into the genetic factors contributing to alcohol addiction.

The fruit fly, *Drosophila melanogaster*, with its accessibility to genetic and molecular analysis, has proven itself as an attractive model system in which to study the genes and pathways that modify acute and chronic behavioral responses to ethanol exposure (Guarnieri and Heberlein, 2003; Wolf and Heberlein, 2003). In response to acute ethanol exposure, flies exhibit behaviors similar to those observed in mammals: low doses of ethanol result in hyperactivity, whereas higher doses result in decreased activity and eventual loss of postural control and sedation (Singh and Heberlein, 2000; Wolf et al., 2002). Unbiased genetic approaches and candidate gene analyses have provided insight into the various molecules and biochemical pathways (Corl et al., 2005; Moore et al., 1998; Park et al., 2000; Rothenfluh et al., 2006; Wen et al., 2005) as well as the neuroanatomical loci (Rodan et al., 2002) that regulate the ethanol response in *Drosophila*. Several of the molecules implicated in ethanol responses in *Drosophila*, such as protein kinase A (PKA), calcium-dependent adenylate cyclase (Moore et al., 1998) and the fly orthologue of neuropeptide Y, NPF (Wen et al., 2005), have been shown to have similar roles in mediating ethanol behaviors in mammals (Maas et al., 2005; Thiele et al., 2002; Thiele et al., 2000), validating the usefulness of *Drosophila* as a valuable tool for identifying candidate genes and pathways underlying the behavioral response to ethanol.

Mitogen-activated protein (MAP) kinase signaling cascades have been shown to play essential roles in regulating a variety of cellular processes, including embryogenesis, cell differentiation and proliferation, cell death, and acute responses to hormones and

environmental stresses (Chen et al., 2001; Pearson et al., 2001). Two of the major MAP kinase cascades, the c-Jun N-terminal kinase (JNK) pathway and the p38 pathway, play important roles in transducing cellular stress responses triggered by stimuli such as osmotic shock, heat shock, and inflammatory cytokines (Pearson et al., 2001). The other major and most well characterized MAP kinase pathway is the extracellular signal-regulated protein kinase (ERK) cascade, activated by growth factors, serum, and cytokines, which has been shown to play major roles not only developmentally in cell proliferation and differentiation, but also postmitotically in regulating synaptic transmission and long-term memory (Mazzucchelli and Brambilla, 2000; Pearson et al., 2001; Sweatt, 2004) as well as circadian rhythms (Kramer et al., 2001). In *Drosophila*, the ERK pathway, and more specifically its activation through the epidermal growth factor (EGF) receptor (EGFR), has been implicated in various phases of development, including the specification of cell fate in the central nervous system, germ band retraction in the embryo, and the development of the retina (Kumar et al., 1998; Perrimon and Perkins, 1997). In recent years, studies *in vitro* and *in vivo* have revealed an intriguing link between ethanol and the mammalian EGFR/ERK pathway, demonstrating that EGFR autophosphorylation and ERK phosphorylation are both inhibited by pharmacologically relevant concentrations of ethanol (Chandler and Sutton, 2005; Ma et al., 2005). While these studies have shown ethanol to act as an inhibitor of EGFR/ERK signaling in both neuronal cell cultures and mammalian brains, the roles of the EGFR/ERK signaling pathway in the behavioral responses to ethanol are unknown.

In order to identify novel molecules and pathways regulating the behavioral response to ethanol in *Drosophila*, we conducted a genetic screen for mutants that

displayed an altered sensitivity to the sedative effects of ethanol. Here, we describe the identification and characterization of loss-of-function mutants in the *happyhour* (*hppy*) gene, which show a marked resistance to ethanol-induced sedation. Conversely, increasing *hppy* expression in the nervous system results in an elevated sensitivity to the sedative effects of ethanol. As mammalian orthologues of *hppy* encode Ste20 family members involved in JNK pathway signaling, we tested whether neuronal perturbation of the JNK pathway affected ethanol sensitivity, but surprisingly, found that neither activation nor inhibition of the JNK pathway had an effect. However, manipulating components in the ERK signaling cascade in the nervous system, specifically the EGFR pathway, strongly affected ethanol sedation sensitivity. We provide genetic evidence that links *hppy* to the EGFR pathway, supporting a model in which *hppy* acts as an inhibitor of the pathway, functioning downstream of the EGFR but upstream of ERK. Thus, we identify *hppy* as a novel regulator of EGFR/ERK signaling, and describe how both *hppy* and the EGFR/ERK pathway modulate behavioral responses to ethanol-induced sedation in *Drosophila*.

Results

hppy mutants display resistance to ethanol-induced sedation

In an effort to identify novel molecules mediating the sedative responses to ethanol in *Drosophila,* we screened a collection of strains carrying the P{GawB} transposable element using a locomotor tracking device (Wolf et al., 2002). When exposed to a relatively high concentration of ethanol (see Methods), *Drosophila* exhibit a fast and transient increase in their locomotor response (a startle response to the smell of

ethanol), followed by a decrease in locomotor activity that is associated with the gradual loss of postural control and, finally, akinesis (sedation) (Wolf et al., 2002). In our screen we isolated one mutant, line 17-51, that displayed resistance to ethanol-induced sedation as measured in the locomotor tracking system (Fig. 1A). Direct observations of sedation responses over a 30 minute ethanol exposure using a modified loss-of righting (LOR) assay (Rothenfluh et al., 2006), revealed that 17-51 mutants do indeed display a marked resistance to ethanol-induced sedation compared to controls (Fig. 1C, D), a phenotype that is observed at all ethanol concentrations tested (Supplementary Fig. 1). This is not simply due to a decrease in ethanol pharmacokinetics in 17-51 flies, as ethanol absorption over various lengths of ethanol exposure were normal in 17-51 flies (Fig. 1B). In addition, 17-51 flies show normal locomotor behavior and negative geotaxis (Supplementary Fig. 2).

Inverse PCR analysis revealed that the P{GawB} element in 17-51 is inserted 10 bp upstream of the first exon of the gene CG7097, and further analysis showed that impaired expression of this gene is responsible for the sedation resistance of 17-51 flies (see below). We decided to name the CG7097 gene *happyhour*, since mutations in the gene result in flies being able to imbibe significantly more alcohol than controls before succumbing to its sedating effects. The transposon inserted in *hppy*$^{17\text{-}51}$ is responsible for the sedation resistance phenotype, as precise excisions of the element reverted the mutant phenotype (Fig. 1C, D). Database searches (http://www.flybase.org) revealed the presence of an additional P-element insertion near the *hppy*$^{17\text{-}51}$ insertion. When tested in the LOR assay, this mutant, *hppy*^{KG5537}, also showed resistance to ethanol-induced sedation (Fig. 1E, F). In addition, complementation assays between *hppy*$^{17\text{-}51}$ and

hppy^{KG5537} revealed that these mutants fail to complement each other's ethanol sedation phenotype (data not shown).

Molecular characterization of the *hppy* locus and *hppy* mutants

Genome analysis of the *happyhour* (CG7097) gene region (http://www.flybase.org) indicated that the gene covers ~48.5 kb, encoding two transcripts, *hppy-RB* and *hppy-RA* (Fig. 2A). Both transcripts, which are generated by alternative splicing of the eighth intron, share the same transcription start site, but the longer 5.1 kb *hppy-RA* transcript contains an additional 800 bp in its ninth exon not contained in the shorter 4.3 kb *hppy-RB* transcript. Our mutant strains contain P element insertions in the 5' gene region of *hppy*. In *hppy*$^{17-51}$, the transposon is inserted in the putative promoter region, 10 bp upstream of the first exon; the *hppy*^{KG5537} transposon is inserted in the first, non-coding exon (Fig. 2A). Both *hppy-RA* and *hppy-RB* transcripts are predicted to encode proteins containing an N-terminal serine/threonine kinase domain and a citron homology domain near the C-terminus (Fig. 2B; Simple Modular Architecture Research Tool: http://smart.embl-heidelberg.de). The closest human homologs of HPPY are members of the germinal center kinase-1 (GCK-1) family of Ste20-related kinases, including GLK (germinal center-like kinase) and GCK itself (Dan et al., 2001; Findlay et al., 2007). GCK-1 family members in other organisms have previously been shown to act as MAP4Ks in the JNK signaling pathway (Chen and Tan, 1999), although *in vitro* studies of CG7097 (*hppy*) have failed to place this putative *Drosophila* MAP4K in the JNK signaling pathway (Findlay et al., 2007).

In order to better understand the effects our P-element insertions have on *hppy* expression, we assessed *hppy* transcript levels in our mutant and control strains by quantitative RT-PCR (QPCR). Using a primer and probe set recognizing both *hppy-RB* and *hppy-RA* transcripts, we found that the relative expression of *hppy* in the $hppy^{17\text{-}51}$ and $hppy^{KG5537}$ mutants was reduced to approximately half that of controls (Fig. 2C). A similar reduction in *hppy* expression was seen in the mutants when using a primer and probe set recognizing specifically the *hppy-RA* transcript (Fig. 2D). We were unable to generate a primer/probe set directed specifically against *hppy-RB*, since the entire sequence of *hppy-RB* is contained within the larger *hppy-RA* transcript. In summary, we have identified two mutations in the *hppy* locus that share a resistance to ethanol-induced sedation and show reduced levels of *hppy* transcripts.

Behavioral rescue of the ethanol resistance of *hppy* mutants

In order to conclusively demonstrate that the sedation resistance observed in *hppy* mutants was due to a decrease in *hppy* expression, we attempted to rescue the mutant behavioral phenotype by expressing a *UAS-hppy* transgene in the *hppy* mutant background. We generated a *UAS-*$hppy^{RB}$ construct by inserting the complete *hppy-RB* cDNA sequence into the pUAST vector (see Methods) and introduced this transgene into $hppy^{17\text{-}51}$ homozygous mutant flies. The $hppy^{17\text{-}51}$ P[GAL4] insertion drives widespread GAL4 expression in tissues including the central nervous system, as visualized with a *UAS-green fluorescent protein* (*UAS-GFP*) reporter transgene (Supplementary Fig. 3A). $hppy^{17\text{-}51}$ homozygous mutant flies carrying the *UAS-*$hppy^{RB1}$ transgene have increased expression of specifically the *hppy-RB* transcript, as assayed by QPCR (Supplementary

Fig. 3B, C). When tested in the LOR assay, these flies (*hppy*$^{17\text{-}51}$/*hppy*$^{17\text{-}51}$; *UAS-hppy*^{RB1}/*UAS-hppy*^{RB1}) displayed wild-type behavior (Fig. 3A, B), indicating complete rescue of the mutant phenotype by *hppy-RB* expression. Partial rescue was also achieved by using a second, more weakly expressed insertion of *UAS-hppy*^{RB} (*UAS-hppy*^{RB2}; Supplementary Fig. 3D, E). Importantly, introducing the *UAS-hppy*^{RB} trangenes into the *hppy*^{KG5537} homozygous mutant background, in which GAL4 is not expressed (see Methods), did not rescue the *hppy*^{KG5537} sedation resistance phenotype (Fig. 3C, D and data not shown). Finally, expression of an innocuous transgene, encoding inactive tetanus toxin light chain in the *hppy*$^{17\text{-}51}$ homozygous mutant flies, failed to rescue the mutant phenotype (data not shown). These data confirm that the reduction in *hppy* expression is responsible for the resistance to ethanol-induced sedation observed in *hppy* mutant flies.

To determine whether expression of *hppy* specifically in the nervous system was sufficient to restore normal ethanol-induced sedation to *hppy* mutant flies, we expressed the *UAS-hppy*^{RB1} transgene in neurons using the *elav-GAL4*^{c155} driver in *hppy*^{KG5537} homozygous mutant flies. Indeed, neuronal expression of *hppy-RB* completely rescued the sedation resistance of *hppy*^{KG5537} flies (Fig. 3E, F). We next asked if neuronal overexpression of *hppy* would lead to enhanced sensitivity to the sedative effects of ethanol, the opposite effect caused by reduced *hppy* expression. Flies expressing the *UAS-hppy*^{RB1} transgene under the control of the pan-neuronal *elav-GAL4*^{c155} driver in an otherwise wild-type background showed a significant increase in sensitivity in the LOR assay (Fig. 3G, H). These data demonstrate that *hppy* functions in neurons to control ethanol-induced sedation, and that the pathway whose function is regulated by *hppy* can enhance or suppress the flies' response to the sedating effects of ethanol.

Perturbations of the JNK signaling pathway do not affect ethanol induced sedation

Since previous work had shown that a human homolog of *hppy*, germinal center kinase (GCK), activates the JNK pathway acting as a MAP4K (Dan et al., 2001; Pombo et al., 1995), we investigated whether perturbation of JNK pathway signaling in *Drosophila* would alter sensitivity to ethanol-induced sedation as measured in the LOR assay. To do this, we expressed various transgenes known to activate or inhibit the JNK pathway using the pan neuronal drivers *elav-GAL4*^{c155} and *elav-GAL4*^{3E1} (*elav-GAL4*^{c155} expresses higher levels of GAL4 than *elav-GAL4*^{3E1}). We found that neither activation nor inhibition of the JNK signaling pathway altered the sensitivity of flies to ethanol-induced sedation, as measured in the ethanol LOR assay. For example, flies neuronally expressing a constitutively activated form of the JNKK *hemipterous* (*hep*) (using a *UAS-hep*^{ACT} transgene) showed wild-type sensitivity (Fig. 4A, B). Similarly, neither neuronal overexpression of the *Drosophila* homolog of JNK, basket (*bsk*, using a *UAS-bsk*^{WT} transgene), nor a dominant-negative form of JNK (using a *UAS-bsk*^{DN} transgene), altered LOR sensitivity (Fig. 4C, D and data not shown). Finally, neuronal overexpression or inhibition of the JNK pathway transcription factor dJUN, through expression of wild-type or a dominant-negative form of dJUN (using *UAS-jun*^{WT} or *UAS-jun*^{DN}, respectively) also failed to affect ethanol-induced sedation behaviors (Supplementary Fig. 4A, B, and Fig. 4E, F). We also tested the effects of perturbing a second major MAPK pathway, the p38 pathway; these manipulations similarly had no significant effect on ethanol-induced sedation (Supplementary Fig. 4C, D and data not shown).

Perturbations of neuronal EGFR signaling alter ethanol sensitivity

Since manipulations of the JNK and p38 pathways failed to alter ethanol sensitivity, we tested the remaining MAPK signaling pathway, the ERK pathway, for its potential role in regulating ethanol sensitivity. We asked if perturbations of the ERK pathway, specifically the EGFR pathway, had an effect on ethanol-induced sedation by driving expression of various EGF pathway transgenes using the pan-neuronal drivers *elav-GAL4*^{c155} or *elav-GAL4*^{3E1}. We also attempted to drive transgene expression with the ubiquitous driver *Tub-GAL4*, but found that this resulted in lethality in all cases except when driving the expression of a secreted form of the EGFR ligand encoded by the *spitz* (*spi*) gene, a condition that produced viable and healthy flies.

Interestingly, we found that manipulations that enhanced EGF signaling at several levels in the pathway potently increased resistance to ethanol-induced sedation, as measured in the LOR assay. Expression of secreted Spitz, by driving expression of a *UAS-spi*^{SEC} transgene using either *Tub-GAL4* or *elav-GAL4*^{c155}, strongly increased resistance to ethanol-induced sedation (Fig. 5A, B, and data not shown). Marked resistance was also produced by driving neuronal expression of a wild-type EGFR transgene (*UAS-egfr*^{WT}, Fig. 5C, D), a gain-of-function Raf MAP3K (*UAS-dRaf*^{GOF}, data not shown), or a constitutively active form of the ERK *rolled* (*rl*) (*UAS-rl*^{ACT}, Fig. 5E, F). Conversely, we found that a P element-induced loss-of-function mutation in *rhomboid-1* (*rho-1*), which codes for an enzyme that activates EGFR signaling through proteolysis of the ligand Spitz (Lee et al., 2001), induces the opposite effect, enhanced sensitivity to ethanol-induced sedation (Fig. 6B, C). In this allele, which carries a P element insertion in the promoter region of *rho-1* (Fig. 6A), mRNA levels are reduced to ~30% of wild-

type as measured by QPCR (Fig. 6D). Taken together, our data strongly support a role for the EGFR pathway in regulating ethanol-induced sedation in *Drosophila*, where inhibition of the pathway leads to enhanced sensitivity to the sedating effects of ethanol, while activation of the pathway leads to the opposite phenotype, resistance to sedation.

Genetic interactions between *hppy* and the EGFR pathway

Based on our observations that enhanced EGFR signaling and loss of *hppy* function both lead to resistance to the sedative effects of ethanol, while reduced EGFR signaling and *hppy* overexpression lead to the opposite effect, enhanced sensitivity, we reasoned that *hppy* may function as an inhibitor of the EGFR pathway. In order to test this hypothesis, we resorted to the fly eye, where the developmental role of EGFR signaling has been thoroughly studied (reviewed in Dominguez et al., 1998). Specifically, we tested whether overexpression of *hppy-RB* could enhance or suppress the rough eye phenotypes induced by expression of EGFR pathway components using the *GMR-GAL4* driver, which drives expression in developing retinal cells (Moses and Rubin, 1991). Expression of *UAS-hppy*^{RB1} under the control of *GMR-GAL4* resulted in an essentially wild-type eye phenotype (compare Fig. 7A and 7B). Overexpressing the EGFR using *UAS-egfr*^{WT} resulted in a very strong rough eye phenotype with prominent blistering in the dorsal anterior section of the eye (Fig. 7C). A rough eye phenotype was also observed when expressing a constitutively active form of the MAPK *rolled*, using the *UAS-rl*^{ACT} transgene, under the control of *GMR-GAL4* (Fig. 7E). We found that *hppy* overexpression was able to suppress the rough eye and blistering phenotypes induced by overexpression of the EGFR (Fig. 7D). In contrast, expression of *hppy* did not affect the rough eye

phenotype caused by expression of activated *rolled* (Fig. 7E, F). Having shown that *hppy* expression could suppress the rough eye phenotype induced by EGFR pathway activation, we asked whether *hppy* expression could enhance the rough eye phenotype caused by the expression of the transcription factor YAN, which acts downstream of *rolled* to inhibit the transcription of EGFR pathway-activated genes (O'Neill et al., 1994). Indeed, while expression of wild-type *yan*, using the *UAS-yan*^{WT} transgene, produced an overall normal looking eye with an orderly arrangement of ommatidia (Fig. 7G), the combined expression of *hppy* and *yan* under *GMR-GAL4* control produced a severe rough and "glossy" eye phenotype in which large swaths of ommatidia were absent (Fig. 7H), showing that *hppy* and *yan* act synergistically to inhibit downstream targets of the EGFR pathway. Taken together, our data implicates a role for *hppy* as an inhibitor of EGFR signaling, acting downstream of the EGFR, but upstream of the MAPK *rolled*.

When expressing various EGFR pathway components with the *GMR-GAL4* driver, we observed that expression of either the dominant negative form of the EGFR (*UAS-egfr*^{DN}) or an activated form of yan (*UAS-yan*^{ACT}), resulted in reduced viability (Supplementary Fig. 5). Interestingly, co-expression of *hppy* (*UAS-hppy*^{RB1}) potently enhanced the lethality of both *GMR-GAL4* driven *UAS-egfr*^{DN} and *UAS-yan*^{ACT} (Supplementary Fig. 5). Taken together, these data serve as strong evidence that *hppy* can interact with the EGFR pathway in its requirement for normal eye development and viability, and is consistent with *hppy* functioning as an inhibitor to the pathway.

Discussion

We identified, through an unbiased genetic screen for *Drosophila* mutants with

altered behavioral sensitivity to ethanol, a mutant in the gene *happyhour* (*hppy*). Loss of function of *hppy* results in increased resistance to ethanol-induced sedation, whereas neuronal overexpression of *hppy* causes the opposite effect, decreased resistance to sedation. *hppy* encodes a presumed MAP4 kinase with homology to GCK. To identify a signaling cascade that *hppy* may regulate, we tested whether neuronal perturbation of the major MAP kinase cascades would affect ethanol sensitivity in *Drosophila*. We found that manipulations that activate or inhibit the JNK or p38 pathways did not affect ethanol-induced sedation. In contrast, perturbations of the ERK pathway activated by the EGFR strongly altered the sensitivity of flies to the sedating effects of ethanol. Specifically, activation of the EGFR pathway in the nervous system resulted in strong resistance to ethanol, whereas inhibition of the pathway induced enhanced sensitivity. Finally, we show that *hppy* can modulate EGFR signaling in a manner that is consistent with it acting as an inhibitor of the pathway, operating downstream of the EGFR but upstream of the ERK Rolled.

happyhour **regulates ethanol-induced sedation in** ***Drosophila***

Utilizing a forward genetic approach to search for *Drosophila* mutants displaying altered responses to ethanol, we identified and characterized two P-element mutants in the CG7097/*happyhour* (*hppy*) gene region. We found that decreased *hppy* expression resulted in decreased sensitivity to the sedative effects of ethanol, as measured in a modified LOR assay, whereas ectopic neuronal expression of *hppy* caused an increased sensitivity to ethanol's sedative effects. Behavioral rescue experiments demonstrated that neuronal expression of *hppy* was sufficient to rescue the *hppy* sedation resistance

phenotype. In addition, we found that complete rescue could be achieved by restoring expression of specifically the *hppy-RB* transcript, suggesting functional redundancy between the *hppy-RB* and *hppy-RA* transcripts or that the *hppy-RA* transcript may not be required for normal ethanol-induced sedation behaviors.

Like its mammalian homologs, the GCK-1 subfamily of Ste20 family kinases, both *hppy* transcripts are predicted to encode proteins that bear N-terminal serine/threonine kinase domains and C-terminal regulatory domains known as citron homology domains. *In vitro* studies of these homologs of *hppy*, including germinal center kinase (GCK) (Pombo et al., 1995), GCK-like kinase (Diener et al., 1997), kinase homologous to SPS1/STE20 (Tung and Blenis, 1997), and hematopoietic progenitor kinase (Kiefer et al., 1996), have revealed that these GCK-1 subfamily members specifically activate the JNK signaling cascade, but not the ERK or p38 MAPK pathways. HPK1 (Hu et al., 1996) and GLK (Diener et al., 1997) have both been shown to phosphorylate MAP3Ks in the JNK signaling pathway, positioning these GCK-1 subfamily kinases as MAP4Ks, situated upstream of the traditional three-tiered MAPK signaling cascade, but downstream of membrane signaling elements. While more distantly related Ste20 group kinases, such as those belonging to the GCK-VIII subfamily of thousand and one (TAO) kinases, have been shown to act as MAP3 kinases, activating both the JNK and the p38 stress-activated MAP kinase cascades (Chen and Tan, 1999; Hutchison et al., 1998; Yustein et al., 2000), until this study there have not been any reports of GCK kinases having a modulatory role on ERK signaling.

In this study we present evidence to show that *hppy*, a presumed MAP4K in the GCK-1 subfamily of Ste20 kinases, can indeed modulate ERK signaling in a manner that

is consistent with it acting as an inhibitor of ERK signaling functioning upstream of ERK itself but downstream of the EGFR. We find that *hppy* can enhance and suppress the rough eye phenotypes brought about by EGFR/ERK perturbations as well as enhance the semi-lethality induced by expression of EGFR/ERK pathway inhibitors. What then is the biochemical mechanism through which *hppy* inhibits EGFR/ERK signaling? The answer to this question is still unknown. However, an *in vitro* study of another GCK-1 subfamily kinase, HPK1, offers up an intriguing possibility (Anafi et al., 1997). In this study, the authors found that HPK1 physically associates with the EGFR adaptor protein Grb2 both in a yeast two-hybrid assay as well as in transfected mammalian cells. EGF stimulation recruits the Grb2/HPK1 complex to the autophosphorylated EGFR. This recruitment then leads to the tyrosine phosphorylation of HPK1, although the functional consequences of this phosphorylation are not known. It will be interesting to determine whether such an association might exist between HPPY and members of the EGFR/ERK signaling cascade, and what consequences this association may have on the signaling of this MAP kinase pathway.

From our experiments we cannot rule out a role for *hppy* in regulating JNK signaling, although the lack of effect of JNK signaling perturbation on behavioral sensitivity to ethanol strongly suggests that *hppy* does not mediate its effects on ethanol-induced sedation through the JNK pathway. We also found that *hppy* mutant flies do not respond differently from controls when exposed to a variety of stress stimuli known to activate the JNK and p38 pathways, including oxidative stress, heat stress, and starvation (data not shown), further supporting the hypothesis that *hppy* is not involved in transducing signals through these stress-activated MAPK pathways. Indeed, *in vitro*

studies in Hela cells support this hypothesis, demonstrating a lack of involvement of *hppy* in mediating JNK activation in response to stress stimuli such as osmotic stress and the protein synthesis inhibitor anisomycin (Findlay et al., 2007). Instead, Findlay et al. offer evidence that *hppy* can act as a nutrient sensor of amino acids and can stimulate phosphorylation of S6 kinase (S6K) through the mammalian target of rapamycin (mTOR) signaling pathway. The authors show that overexpression of wild-type *hppy*, but not a kinase-inactive mutant, can induce this S6K phosphorylation, demonstrating that *hppy* can in fact function as a kinase (Findlay et al., 2007). Interestingly, the mTOR signaling pathway, a key regulator of cell growth, is itself intimately regulated by inputs from the ERK signaling pathway (Sarbassov et al., 2005), suggesting the attractive possibility that *hppy* may exert its stimulatory role on the mTOR pathway via its effects on EGFR/ERK pathway signaling.

The EGFR/ERK pathway mediates ethanol-induced sedation in *Drosophila*

The ERK signaling cascade has traditionally been studied for its roles in regulating a variety of developmental processes, including cell division, survival, and differentiation (Chen et al., 2001; Pearson et al., 2001). More recent research, however, has revealed that the ERK pathway also plays important roles in mediating synaptic plasticity in post-mitotic neurons (Mazzucchelli and Brambilla, 2000; Sweatt, 2004). For instance, establishment of long-term potentiation requires the activation of ERK, and inhibition of ERK signaling has been shown to disrupt both hippocampal- and amygdala-dependent learning (Atkins et al., 1998; Brambilla et al., 1997; Selcher et al., 1999). In addition, the EGFR/ERK pathway has been implicated in the regulation of circadian

rhythms via its activation by transforming growth factor-a in the suprachiasmatic nucleus (Hao and Schwaber, 2006; Kramer et al., 2001). Recently, an interesting literature has documented the inhibitory effects of ethanol on the EGFR/ERK pathway in neurons. Ethanol administration inhibits EGFR and ERK phosphorylation both in neuronal cell cultures (Chandler and Sutton, 2005; Kalluri and Ticku, 2003; Ma et al., 2005) as well as in mouse and rat brains (Kalluri and Ticku, 2002; Sanna et al., 2002). These results beg the question: what role might the EGFR/ERK pathway play in regulating the acute behavioral response to ethanol?

In this paper, we move towards answering this question by uncovering a previously undocumented role for the EGFR/ERK pathway in mediating the behavioral responses to ethanol in *Drosophila*. Neuronal manipulations that activate the EGFR/ERK pathway result in decreased sensitivity to the sedative effects of ethanol, whereas inhibition of the pathway results in increased sensitivity to ethanol-induced sedation. These effects were seen through manipulations of various components of the EGFR/ERK pathway, including ERK itself, the MAP3K (dRaf), the EGFR, the EGF receptor ligand Spitz, and the enzyme, Rhomboid-1, that processes Spitz into its active form. In contrast, we find no evidence for the other two major MAPK pathways, the JNK and p38 pathways, in mediating the sedative response to ethanol, suggesting a specific role for the ERK pathway. The EGFR/ERK pathway joins other growth factor pathways, such as the insulin, glial cell line-derived neurotrophic factor (GDNF), and brain-derived neurotrophic factor (BDNF) pathways, as regulators of the behavioral response to ethanol (Janak et al., 2006).

The pathways through which the EGFR/ERK cascade detects ethanol signals and

how it might transduce those signals into a behavioral response remain unknown. The ERK cascade is activated through a variety of sources, including growth factors, serum, and ligands for heterotrimeric G protein-coupled receptors (Chen et al., 2001). Interestingly, recent studies have implicated a role for both $GABA_A$ and NMDA receptors in ethanol-mediated inhibition of ERK *in vitro*, suggesting that ethanol may influence ERK activation state via signaling through these receptors (Kalluri and Ticku, 2002; Kalluri and Ticku, 2003). The targets of ERK signaling are multitudinous, and our knowledge of the substrates of ERK signaling is ever expanding. In addition to phosphorylating and activating various transcription factors, ERK also targets various protein kinases, voltage-gated ion channels, and second messenger systems such as cytosolic phospholipase A2 (Atkins et al., 1998; Chen et al., 2001). One alluring target of ERK signaling is the phosphodiesterase (PDE) 4D3, a cyclic adenosine monophosphate (cAMP)-specific phosphodiesterase that negatively regulates cAMP/PKA signaling by degrading intracellular cAMP (Chen et al., 2001). The cAMP signaling pathway, in turn, has been shown to regulate the ERK pathway, with inhibition of ERK signaling being induced by C-RAF phosphorylation by PKA (Dumaz and Marais, 2005). Various studies in both *Drosophila* and mammals have demonstrated a role for the cAMP/PKA signaling cascade in mediating the behavioral responses to ethanol (Moore et al., 1998; Park et al., 2000; Thiele et al., 2000; Maas et al., 2005), raising the exciting possibility that "crosstalk" between these two conserved pathways may be integral to regulating ethanol induced sedation behaviors.

Experimental Procedures

Genetic Screen and Selection of Controls: Approximately 850 P[GAL4] insertions (carrying the GawB element) in the *w* Berlin genetic background were screened as homozygotes in the booz-o-mat at a 100U ethanol/50U air concentration as described below. After each day of screening, each fly line's ethanol-induced locomotor tracking profile was compared to the mean tracking profile of the day. Lines were judged to have a mutant locomotor tracking phenotype if they differed by at least 2 standard deviations from the mean at two or more consecutive time points. The 11 lines that retained their mutant phenotypes after two or more retests were selected for backcrossing for five generations to the parental *w* Berlin strain and retested after outcrossing. In addition, 32 control lines, including line 8-165 used in this paper, exhibiting tracking profiles similar to the mean tracking profile of the screened population over multiple days, were also backcrossed to the parental *w* Berlin strain in order to generate a set of control lines. Of the 11 outcrossed mutant lines, five, including 17-51 (*hppy*) retained their mutant phenotypes. The characterization of the other 4 mutant lines is discussed in Chapter 2 of this dissertation.

Fly Stocks

Flies were raised on standard cornmeal and molasses food at 25°C and 70% relative humidity. All experiments were performed on 2-5 day old males at 20°C, utilizing 25 males for each behavioral run. All genotypes were tested across multiple days. All lines tested in behavioral experiments were in the same genetic background (*w* Berlin) with the exception of *UAS-spi*^{SEC} and *UAS-dRaf*^{GOF}, which were in the w^{1118} background isogenic

for chromosomes II and III. Lines 17-51 (*hppy*) and control line 8-165 were obtained through the screen described above. Excisions of 17-51 were carried out through standard genetic crosses using the {delta 2-3} jump-starter chromosome (Robertson et al., 1988). *Elav-GAL4*^{c155}, *Tub-GAL4*, *GMR-GAL4*, and *hppy*^{KG5537} flies were obtained from the *Drosophila* Stock Center (Bloomington, Indiana). *Elav-GAL4*^{3E1} flies were obtained from S. Sweeney (Davis et al., 1997). Mutant *rho-1*^{A0544} (Iks) was obtained from Tim Tully (Dubnau et al., 2003). JNK and p38 pathway lines used include *UAS-bsk*^{WT} (Boutros et al., 1998), *UAS-bsk*^{DN} (Adachi-Yamada et al., 1999a), *UAS-hep*^{ACT} (Weber et al., 2000), *UAS-jun*^{DN} (Eresh et al., 1997), *UAS-jun-RNAi* (Jindra et al., 2004), *UAS-jun*^{WT} (Eresh et al., 1997) and *UAS-dp38b*^{DN} (Adachi-Yamada et al., 1999b). EGF pathway lines used include *UAS-spi*^{SEC} (Ghiglione et al., 2003), *UAS-egfr*^{WT} (Freeman, 1996), *UAS-egfr*^{DN} (Freeman, 1996), *UAS-dRaf*^{GOF} (Brand and Perrimon, 1993), *UAS-rl*^{ACT} (Ciapponi et al., 2001), *UAS-yan* (Rebay and Rubin, 1995), and *UAS-yan*^{ACT} (Rebay and Rubin, 1995).

Locomotor Tracking Assay

Locomotor tracking assays were performed in the "booz-o-mat" as described previously (Wolf et al., 2002). Briefly, twenty-five 2-4 day old males of each genotype were introduced to the chambers in the booz-o-mat. Flies were allowed to equilibrate in humidified air for 10 minutes before digital camera filming commenced. The motion of the flies was then recorded for 2 minutes in humidified air, followed by 21 minutes in an ethanol/air mixture of 100 U ethanol/50U air or 110 U ethanol/40 U air. Films were then analyzed with a modified version of DIAS 3.2 (Solltech, Oakdale, IA) and the average speed of the flies was plotted as a function of time.

Ethanol Sedation Assay

Twenty-five 2-4 day old males of each genotype were introduced to the chambers in the booz-o-mat. After being given 12 minutes of humidified air to equilibrate to the apparatus, the flies were given a continuous stream of ethanol vapors (110U EtOH / 40U air) for 30 minutes. During this thirty period time period, flies were visually assayed for sedation at 10 time points. At each time point, the flies were given a mechanical stimulus (each tube containing flies was twirled within each booz-o-mat chamber) and the numbers of flies that were lying immobile at the bottom of each tube post twirling were scored as being sedated. The assayer was blinded to the identity of the genotypes for the course of the sedation assay. The time to 50% sedation was determined by linear interpolation between the two points flanking the median for each set of 25 flies tested, as previously described (Rothenfluh et al., 2006).

Ethanol Absorption Assay

Twenty-five flies of each genotype were exposed in quadruplicate to an ethanol/humidified air mixture of 100/50 U for 0, 5, 10, 15, or 20 minutes in perforated test tubes in the booz-o-mat apparatus. Following exposure to ethanol, flies were frozen on dry ice and homogenized in 500 μl of 50 mM Tris-HCl (pH 7.5). Ethanol assays were then performed on the fly homogenates as previously described (Moore et al., 1998).

Scanning Electron Microscopy

Scanning electron microscopy was carried out as described previously (Kimmel et al., 1990). All images were taken at 130x magnification.

Molecular Biology

The genomic DNA flanking the 17-51 (*hppy*) insertion was isolated using inverse PCR. Comparisons with the genome sequence of *Drosophila* on Flybase (www.flybase.org) revealed that the insertion was located 10 base pairs upstream of the first exon of CG7097. This finding was confirmed by PCR analysis. The *UAS-hppy* transgene was generated by cloning the EST RH10407, which encodes the full length CG7097-RB transcript, into the pUAST vector (Brand and Perrimon, 1993). The transgene was injected into *w* Berlin flies.

Real-time quantitative RT-PCR

Flies 2-4 days old were collected, frozen immediately in liquid nitrogen, then stored at -80°C. RNA was extracted from whole flies by homogenizing the flies in Trizol Reagent (Invitrogen). Quantitative RT-PCR was performed as described in Tsai et al. (2004). The amplification primers and probe recognizing both *CG7097*-RA and *CG7097*-RB transcripts were: *CG7097*-RA&RB-For, CAGCGTTTTGGCATTCCATAA; *CG7097*-RA&RB-Rev, CGTCACCTCGCCATTGC; and *CG7097*-RA&RB-Probe, ATGCAGGGAAAGTC. The amplification primers and probe recognizing specifically the *CG7097*-RA transcript were: *CG7097*-RA-For, GTTGGCCACATGGGTATGG; *CG7097*-RA-Rev, GGTGCGCAGGTTGGACAT, and *CG7097*-RA-Probe, ATTTGGCATGGGTCTC. The amplification primers and probes for *rhomboid-1*

expression analysis were obtained from Applied Biosystems (http://www.appliedbiosystems.com/). The assay IDs for the primer/probe sets used are: Dm01821933_g1 (rhomboid) and Dm02151827_g1 (Rpl32) (Fig. 6D), as well as Dm01821932_m1 (rhomboid-1, more 5') and Dm02151827_g1 (Rpl32) (data not shown).

Statistics

Statistical significance was established using either Student's *t*-tests assuming equal variance or one-way analysis of variance (ANOVA) tests followed by post-hoc Newman-Keuls testing using GraphPad Prism software, Version 4 (Graphpad, San Diego, CA). Error bars in all experiments represent the standard error of the mean (SEM).

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Figure legends

Figure 1. *hppy* mutants display increased resistance to ethanol-induced sedation. (**A**) *hppy*$^{17\text{-}51}$ flies show increased resistance to ethanol induced sedation as measured by the locomotor tracking system. The speed of *hppy*$^{17\text{-}51}$ flies and control line 8-165 (Ctl) flies were plotted as a function of time. Ethanol exposure (110 U ethanol/40 U air; 110/40 E/A) commenced at 0 min and was continuous thereafter. *hppy*$^{17\text{-}51}$ flies show increased locomotor speed compared to the control (*Ctl*, line 8-165) at 15, 17.5 and 20 minutes. Individual time points analyzed by Student's unpaired *t*-tests assuming equal variance, with the critical *p* value adjusted to $\alpha = 0.01$, revealed significant differences at 20 minutes (p=0.0010), 17.5 minutes (p=0.0018), and 15 minutes (p=0.0007), but not at 12.5 minutes (p=0.0102) or at 10 minutes (p=0.2089). n=6. (**B**) *hppy*$^{17\text{-}51}$ flies do not have alterations in ethanol pharmacokinetics. Ethanol levels were measured in extracts of *hppy*$^{17\text{-}51}$ and control flies (see Methods). Ethanol exposure is continuous starting at time = 0. Two-way ANOVA analysis failed to reveal a significant difference between genotypes (p=0.1480). n=4. (**C, D**) *hppy*$^{17\text{-}51}$ flies show increased resistance to ethanol-induced sedation as measured by the loss of righting (LOR) assay (**C**), while excision of the P-element in *hppy*$^{17\text{-}51}$ (exc. 3 and 16) reverts the sedation resistance phenotype. Ethanol exposure (110/40 E/A) commenced at 0 min and was continuous thereafter. n=8. (**D**) The median sedation time (ST50) – the time required for half of the ethanol-exposed flies to show LOR – was calculated by linear interpolation (see Methods). One-way ANOVA followed by post-hoc Newman-Keuls testing revealed significant differences between the ST50 of *hppy*$^{17\text{-}51}$ and control flies (p<0.001) whereas the ST50 values of the two excisions were not significantly different from that of the control line (p>0.05). n=8.

(**E, F**) *hppy*^{KG5537} flies, which also carry a P-element insertion in the *hppy* gene locus, also show resistance to ethanol-induced sedation. (**E**) Sedation profiles and (**F**) ST50 values were calculated for *hppy*$^{17-51}$, control, and *hppy*^{KG5537}. One-way ANOVA of the ST50 values followed by post-hoc Newman-Keuls testing revealed a significant difference between genotypes ($p<0.0001$), with both *hppy* insertions significantly more resistant to ethanol induced sedation than the control ($p<0.001$ for each comparison). n=12. In this an all figures, error bars represent the standard error of the mean (SEM). Stars denote statistical significance.

Figure 2. Molecular characterization of the *hppy* gene region (CG7097).

(**A**) Cartoon of the *hppy* transcription unit. Exons are shown as boxes. M indicates the translation start site, while * indicates the translation stop codon. Blue arrows indicate the regions amplified for quantitative RT-PCR analysis. The structures of the two transcripts, *hppy-RA* and *hppy-RB*, are diagrammed, as well as the insertion sites of *hppy*$^{17-51}$ and *hppy*^{KG5537}. (**B**) A schematic of the HPPY protein. Both proteins encoded by *hppy-RA* and *hppy-RB* contain a serine/threonine kinase near the N-terminus and a citron homology domain near the C-terminus. (**C, D**) Expression of *hppy* is reduced in *hppy* mutants. RNA was isolated from whole flies and quantitative RT-PCR (QPCR) was performed as described (see Methods). Analysis was also performed on heads and bodies separately and similar results were found (data not shown). Relative mRNA levels are expressed as fold increase over *w* Berlin (*wB*) control RNA. All QPCR experiments on whole flies were repeated with essentially identical results (data not shown). (**C**) QPCR on whole flies using a primer/probe set recognizing both *hppy-RA* and *hppy-RB* transcripts. One-

way ANOVA revealed a significant difference between genotypes (p<0.0001). Post-hoc Newman-Keuls analysis revealed a significant difference between *hppy*$^{17\text{-}51}$ and both *wB* and Ctl (p<0.001) and between *hppy*^{KG5537} and both controls (p<0.001 for each comparison). n=3. (**D**) QPCR on whole flies using a primer/probe set recognizing specifically the *hppy-RA* transcript. One-way ANOVA revealed a significant difference between genotypes (p<0.0001). Post-hoc Newman-Keuls analysis revealed a significant differences between *hppy*$^{17\text{-}51}$ and both controls (p<0.001) and between *hppy*^{KG5537} and both controls (p<0.001). n=3.

Figure 3. Phenotypic rescue and overexpression of *hppy*.

(**A, B**) The *hppy*$^{17\text{-}51}$ sedation resistance phenotype can be rescued by expression of the *UAS-hppy*^{RB1} transgene in the *hppy*$^{17\text{-}51}$ homozygous mutant background. (**A**) Sedation profiles and (**B**) ST50 values are shown. One-way ANOVA of the ST50 values revealed a significant difference between genotypes (p<0.0001). Post-hoc Newman-Keuls analysis revealed a significant difference between *hppy*$^{17\text{-}51}$; *UAS-hppy*^{RB1} and *hppy*$^{17\text{-}51}$ (p<0.001). *hppy*$^{17\text{-}51}$*; UAS-hppy*^{RB1} was not significantly different from Control (p>0.05) or *UAS-hppy*^{RB1} (p>0.05). n=8-12. (**C, D**) Introduction of the *UAS-hppy*^{RB1} transgene into the *hppy*^{KG5537} mutant background, which lacks GAL4 activity, does not rescue the *hppy*^{KG5537} sedation resistance phenotype. (**C**) Sedation profiles and (**D**) ST50 values are shown. One-way ANOVA of the ST50 values revealed a significant difference between genotypes (p<0.0001). Post-hoc Newman-Keuls analysis revealed a difference between *hppy*^{KG5537}*; UAS-hppy*^{RB1} and Control (p<0.001) as well as between *hppy*^{KG5537}; *UAS-hppy*^{RB1} and *UAS-hppy*^{RB1} (p<0.001). *hppy*^{KG5537}*; UAS-hppy*^{RB1} was not significantly

different from *hppy*^{KG5537} (p>0.05). n=8. (**E, F**) Pan-neuronal expression of *UAS-hppy*^{RB1} under the control of the *elav-GAL4*^{c155} driver rescues the sedation resistance phenotype of *hppy*^{KG5537} flies. (**E**) Sedation profiles and (**F**) ST25 values are shown. One-way ANOVA of the ST25 values revealed a significant difference between genotypes (p<0.0001). Post-hoc Newman-Keuls analysis revealed a difference between *elav-GAL4*^{c155}*; hppy*^{KG5537}*; UAS-hppy*^{RB1}/+ and *hppy*^{KG5537} (p<0.001), *elav-GAL4*^{c155}*; hppy*^{KG5537} (p<0.001), and *hppy*^{KG5537}*; UAS-hppy*^{RB1}/+ (p<0.001). *elav-GAL4*^{c155}*; hppy*^{KG5537}*; UAS-hppy*^{RB1}/+ was not significantly different from Control (p>0.05). n=8. (**G, H**) Neuronal overexpression of *UAS-hppy*^{RB1} using the *elav-GAL4*^{c155} driver increases sensitivity to ethanol-induced sedation. (**G**) Sedation profiles and (**H**) ST50 values are shown. One-way ANOVA of the ST50 values revealed a significant difference between genotypes (p<0.0001). Post-hoc Newman-Keuls analysis revealed a difference between *elav-GAL4*^{c155}; *UAS-hppy*^{RB1}/+ and *elav-GAL4*^{c155} (p<0.001) and between *elav-GAL4*^{c155}; *UAS-hppy*^{RB1}/+ and *UAS-hppy*^{RB1}/+ (p<0.001). n=8.

Figure 4. Perturbation of JNK signaling does not alter ethanol sensitivity as measured in the ethanol LOR assay.

(**A, B**) Flies expressing a constitutively active JNKK, *UAS-hep*^{ACT}, under the control of the pan-neuronal driver *elav-GAL4*^{3E1} have an ST50 similar to those of controls. (**A**) Sedation profiles and (**B**) ST50 values are shown. One-way ANOVA of the ST50 values failed to reveal a significant difference between genotypes (p=0.0618). n=8. (**C, D**) Flies expressing wild-type JNK, *UAS-bsk*^{WT}, under the control of the pan-neuronal driver *elav-GAL4*^{c155} have an ST50 similar to those of controls. (**C**) Sedation profiles and (**D**) ST50

values are shown. One-way ANOVA of the ST50 values failed to reveal a significant difference between genotypes (p=0.0955). n=8. (**E, F**) Flies expressing a dominant negative form of the transcription factor dJun, *UAS-jun*^{DN}, under the control of the pan-neuronal driver *elav-GAL4*^{c155} have an ST50 similar to those of controls. (**E**) Sedation profiles and (**F**) ST50 values are shown. One-way ANOVA of the ST50 values failed to reveal a significant difference between genotypes (p=0.2254). n=8.

Figure 5. Activation of EGFR/ERK signaling in neuronal tissues decreases ethanol sensitivity as measured in the ethanol LOR assay.

(**A, B**) Flies expressing a secreted form of the EGFR ligand Spitz, *UAS-spi*^{SEC}, under the control of the pan-organismal driver *Tub-GAL4* are resistant to ethanol-induced sedation. (**A**) Sedation profiles and (**B**) ST50 values are shown. One-way ANOVA of the ST50 values revealed a significant difference between genotypes (p<0.0001). Post-hoc Newman-Keuls analysis revealed a significant difference between *Tub-GAL4; UAS-spi*^{SEC} and *Tub-GAL4* (p<0.01) as well as between *Tub-GAL4; UAS-spi*^{SEC} and *UAS-spi*^{SEC} (p<0.001). n=8. (**C, D**) Flies expressing a wild-type form of the EGFR, *UAS-egfr*^{WT}, under the control of the pan-neuronal driver *elav-GAL4*^{c155} are resistant to ethanol-induced sedation. (**C**) Sedation profiles and (**D**) ST50 values are shown. One-way ANOVA of the ST50 values revealed a significant difference between genotypes (p<0.0001). Post-hoc Newman-Keuls analysis revealed a significant difference between *elav-GAL4*^{c155}*; UAS-egfr*^{WT} and *elav-GAL4*^{c155} (p<0.001) as well as between *elav-GAL4*^{c155}*; UAS-egfr*^{WT} and *UAS-egfr*^{WT} (p<0.001). n=8. (**E, F**) Flies expressing a constitutively active form of the ERK *rolled*, *UAS-rl*^{ACT}, under the control of the pan-

neuronal driver *elav-GAL4*^{c155} are resistant to ethanol-induced sedation. (**E**) Sedation profiles and (**F**) ST50 values are shown. One-way ANOVA of the ST50 values revealed a significant difference between genotypes (p=0.0002). Post-hoc Newman-Keuls analysis revealed a significant difference between *elav-GAL4*^{c155}*; UAS-rl*^{ACT} and *elav-GAL4*^{c155} (p<0.01) as well as between *elav-GAL4*^{c155}*; UAS-rl*^{ACT} and *UAS-rl*^{ACT} (p<0.001). n=7-8.

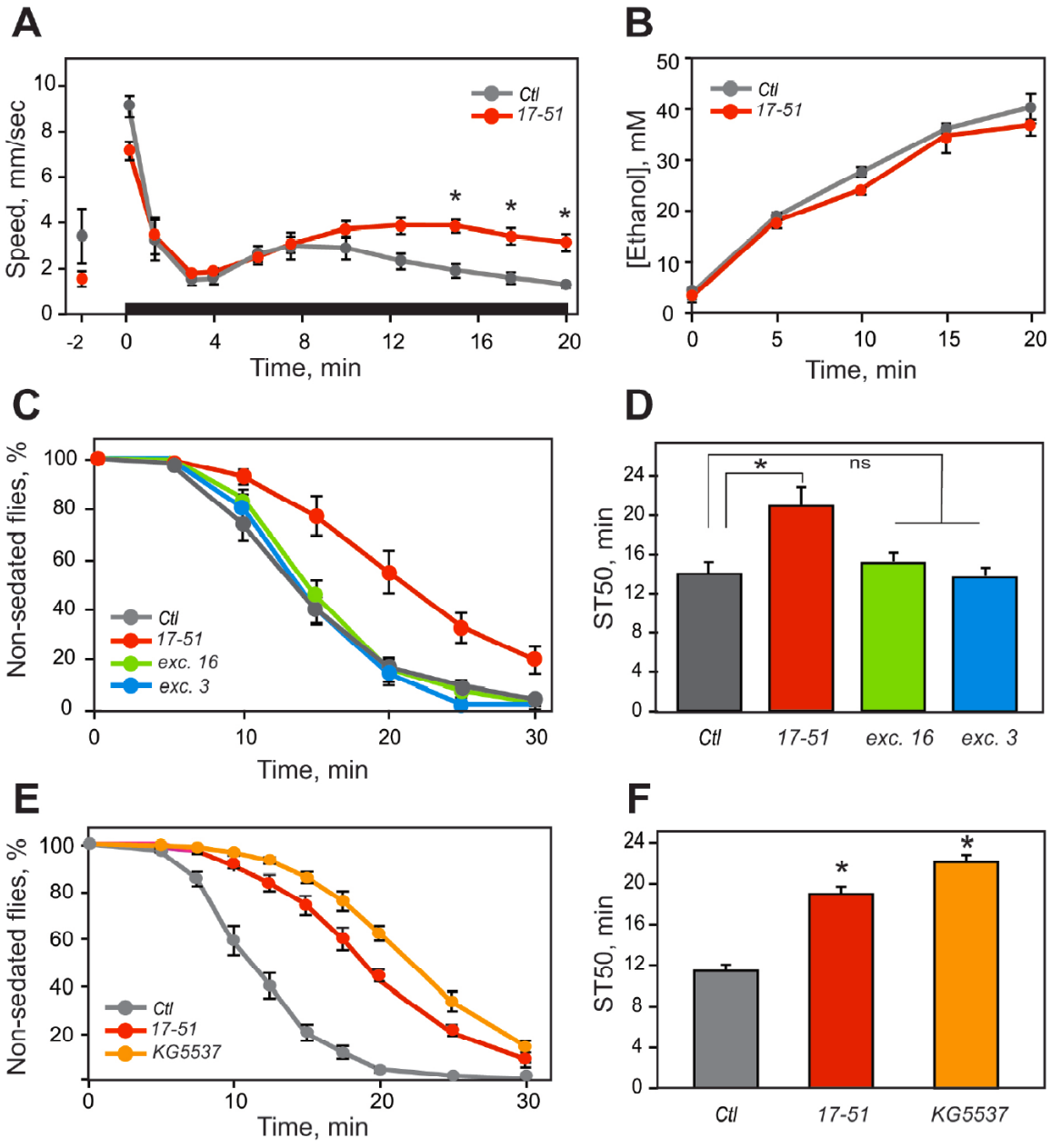
Figure 6. The P-element induced loss-of-function *rhomboid-1* mutant, *rho-1*^{A0544}, displays enhanced sensitivity to ethanol-induced sedation.

(**A**) A diagram of the *rhomboid-1* gene region. Exons are shown as boxes. The insertion site of the P-element A0544 is indicated. Blue arrows delineate the region amplified for QPCR analysis. (**B, C**) *rho-1*^{A0544} flies are sensitive to ethanol-induced sedation as measured in the LOR assay. (**B**) Sedation profiles and (**C**) ST50 values of *rho-1*^{A0544} flies and control flies are shown. Student's unpaired *t*-test assuming equal variance of the ST50 values revealed a significant difference between genotypes (p<0.0001). n=8. (**D**) Expression of *rhomboid-1* is reduced in *rho-1*^{A0544} mutants. RNA was isolated from heads and QPCR was performed as described (see Methods). Relative mRNA levels are expressed as fold increase over the control (line 8-165) RNA. Student's unpaired *t*-test assuming equal variance revealed a significant difference between genotypes (p=0.0102). Quantitative RT-PCR was repeated using a second set of primers recognizing a more 5' region of the *rhomboid-1* gene with similar results (data not shown). n=3.

Figure 7. Genetic interactions between EGFR pathway and *hppy* in the fly eye.

GMR-GAL4 driven *hppy-RB* expression suppresses and enhances the rough eye phenotype caused by overexpression of EGFR and Yan, respectively; *hppy* overexpression does not affect the rough eye of flies expressing an activated *rolled* transgene. Scanning electron micrographs of adult eyes of the following genotypes: (**A**) *GMR-GAL4*, (**B**) *GMR-GAL4;UAS-hppy*^{RB1}, (**C**) *GMR-GAL4;UAS-egfr*^{WT}, arrow points to blister, (**D**) *GMR-GAL4;UAS-egfr*^{WT}*;UAS-hppy*^{RB1}, (**E**) *GMR-GAL4;UAS-rl*^{ACT}, (**F**) *GMR-GAL4;UAS-rl*^{ACT}*;UAS-hppy*^{RB1}, (**G**) *GMR-GAL4;UAS-yan*, (**H**) *GMR-GAL4;UAS-yan;UAS-hppy*^{RB1}. Flies are heterozygous for all transgenes. Anterior is to the right.

Figure 1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Time, min
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Non-sedated flies, %
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Time, min
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17-51
exc. 16
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Non-sedated flies, %
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17-51
KG5537

Figure 2

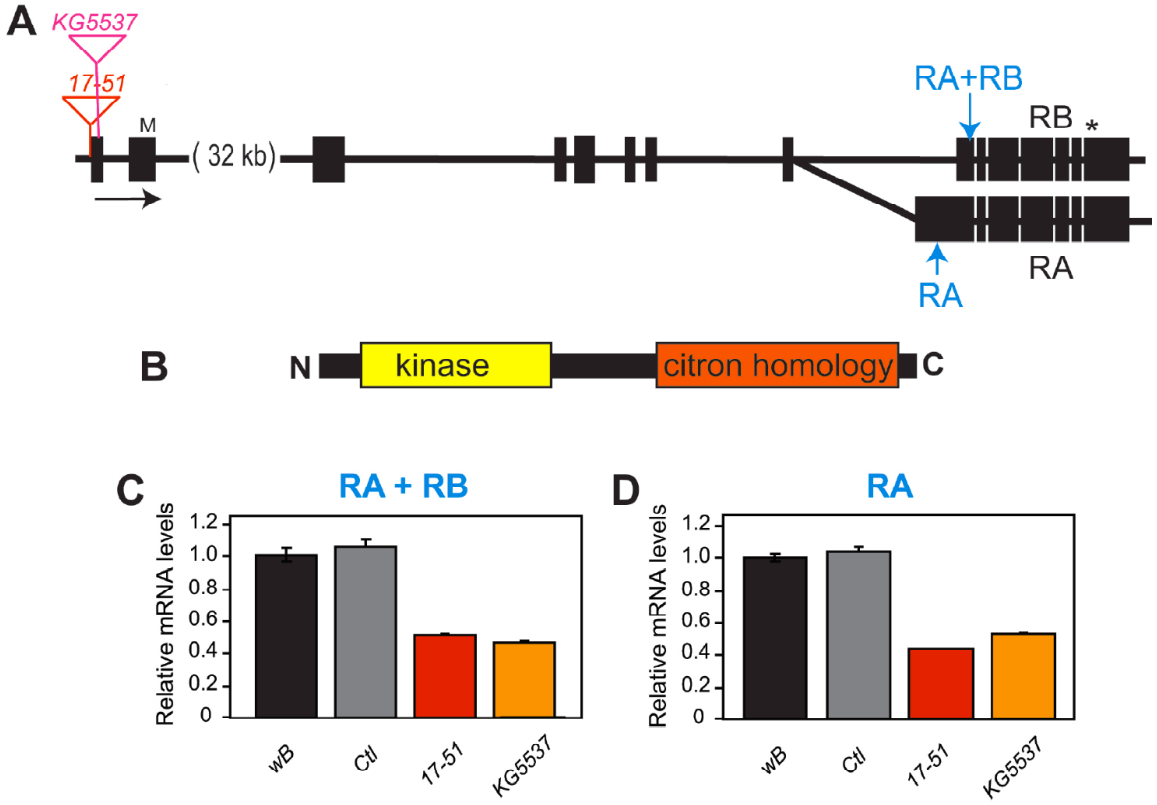
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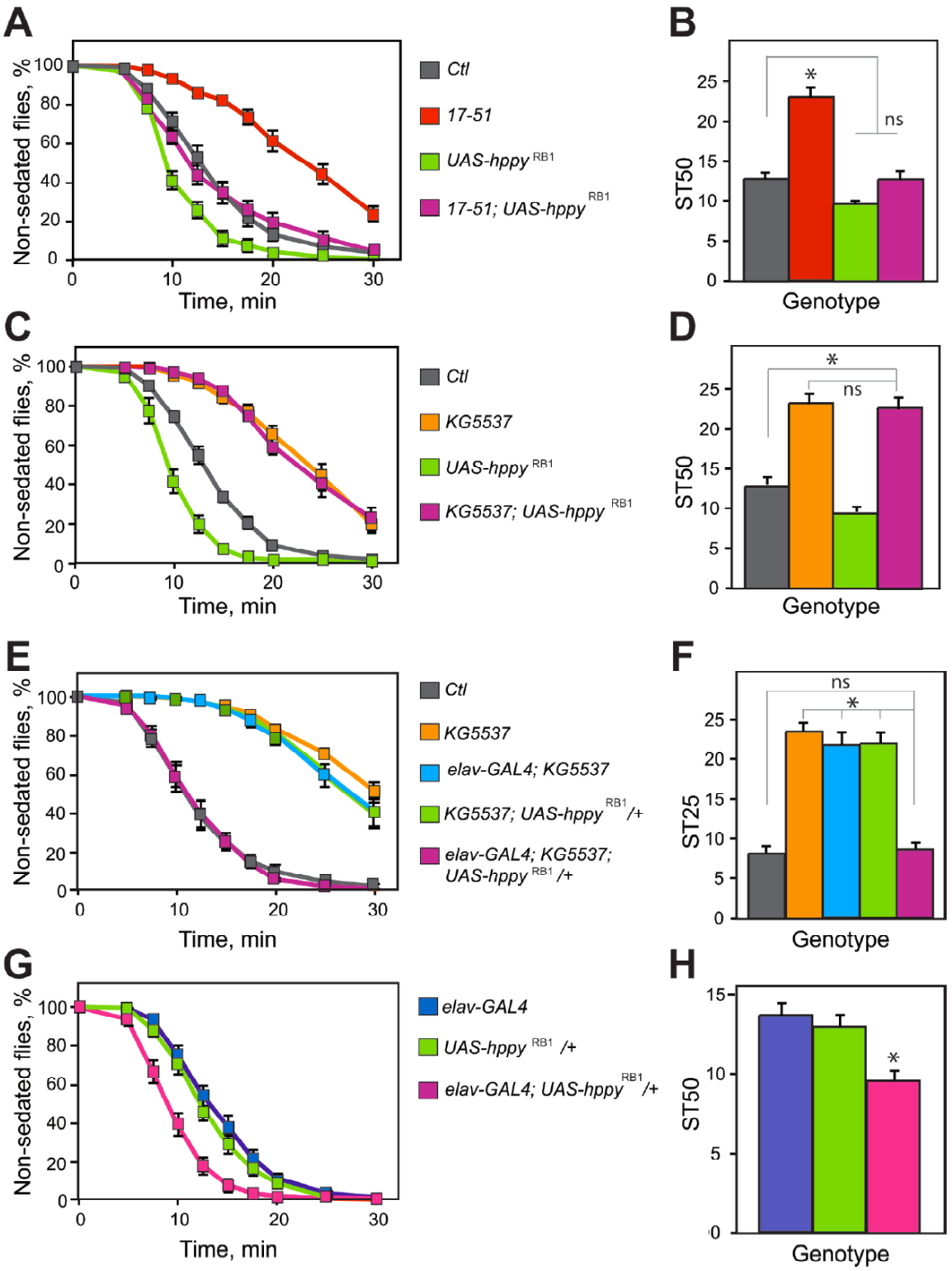
Figure 3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Non-sedated flies, %
Time, min
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UAS-hppy RB1
17-51; UAS-hppy RB1
B
ST50
Genotype
*
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C
Non-sedated flies, %
Time, min
Ctl
KG5537
UAS-hppy RB1
KG5537; UAS-hppy RB1
D
ST50
Genotype
*
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E
Non-sedated flies, %
Time, min
Ctl
KG5537
elav-GAL4; KG5537
KG5537; UAS-hppy RB1/+
elav-GAL4; KG5537; UAS-hppy RB1/+
F
ST25
Genotype
ns
*
G
Non-sedated flies, %
Time, min
elav-GAL4
UAS-hppy RB1 /+
elav-GAL4; UAS-hppy RB1/+
H
ST50
Genotype
*

Figure 4

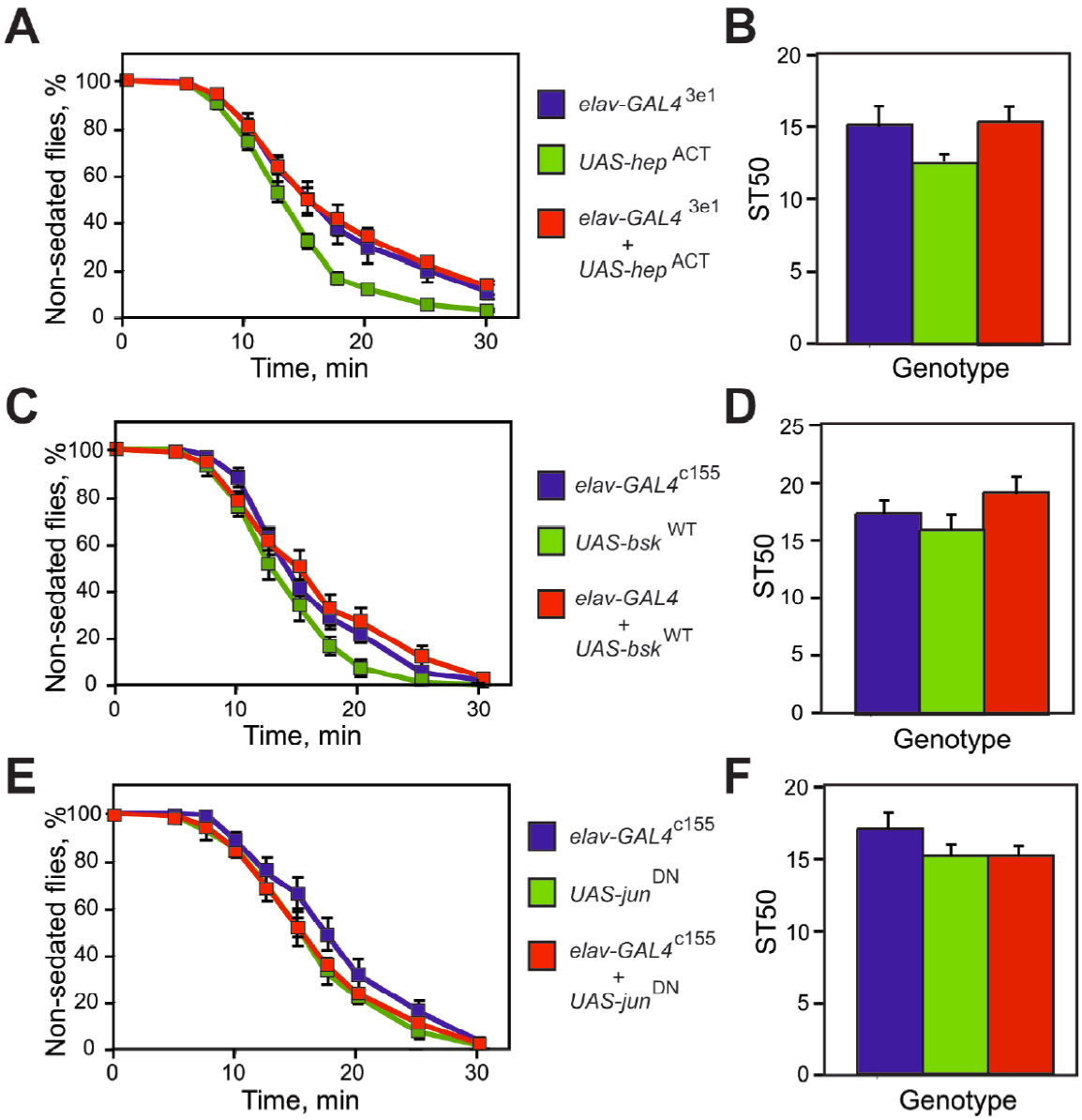

A
Non-sedated flies, %
Time, min
elav-GAL4 3e1
UAS-hep ACT
elav-GAL4 3e1 + UAS-hep ACT
B
ST50
Genotype
C
Non-sedated flies, %
Time, min
elav-GAL4 c155
UAS-bsk WT
elav-GAL4 + UAS-bsk WT
D
ST50
Genotype
E
Non-sedated flies, %
Time, min
elav-GAL4 c155
UAS-jun DN
elav-GAL4 c155 + UAS-jun DN
F
ST50
Genotype

Figure 5

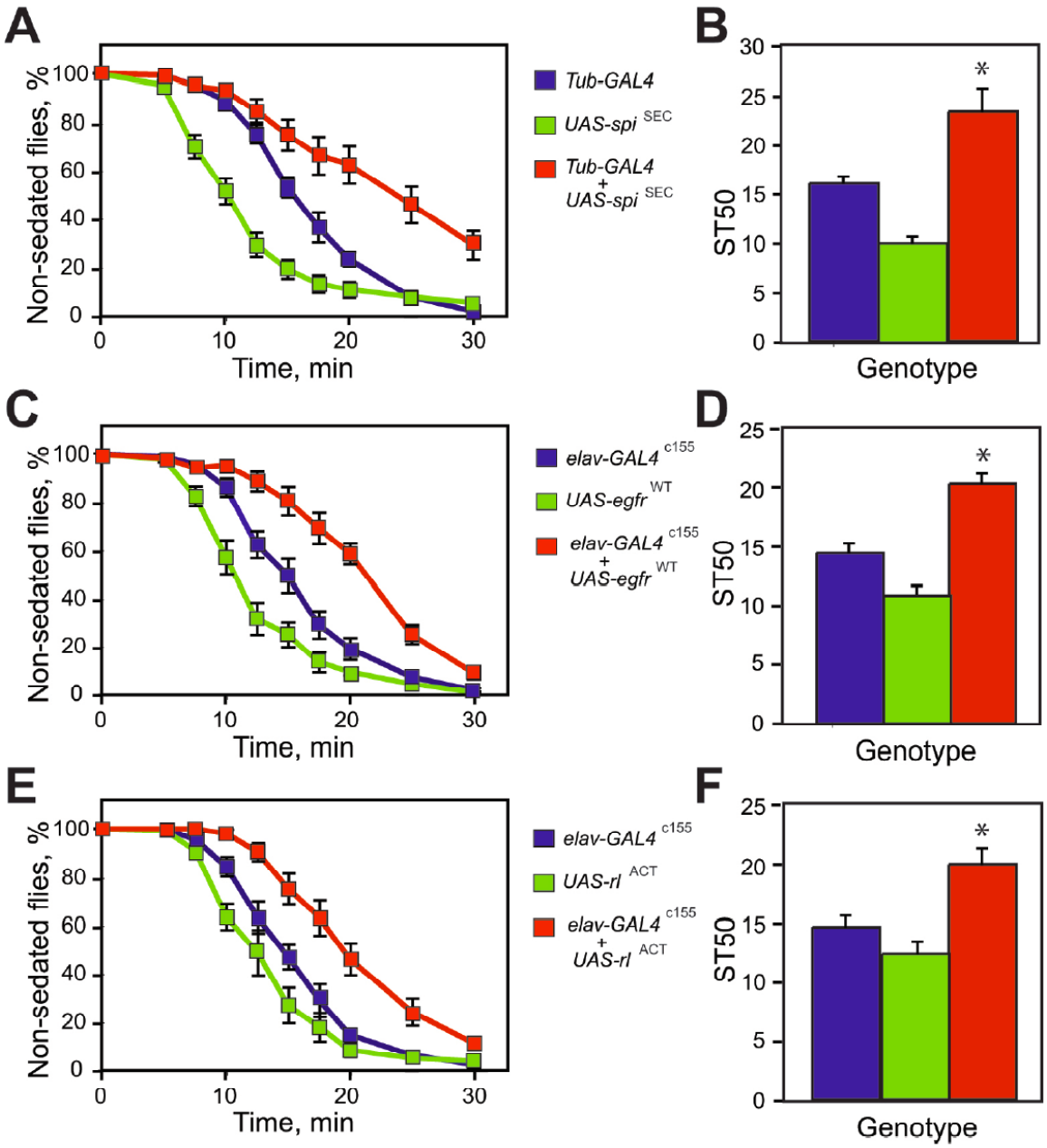

A
Non-sedated flies, %
Time, min
Tub-GAL4
UAS-spi SEC
Tub-GAL4 + UAS-spi SEC
B
ST50
Genotype
C
elav-GAL4 c155
UAS-egfr WT
elav-GAL4 c155 + UAS-egfr WT
D
E
elav-GAL4 c155
UAS-rl ACT
elav-GAL4 c155 + UAS-rl ACT
F

Figure 6

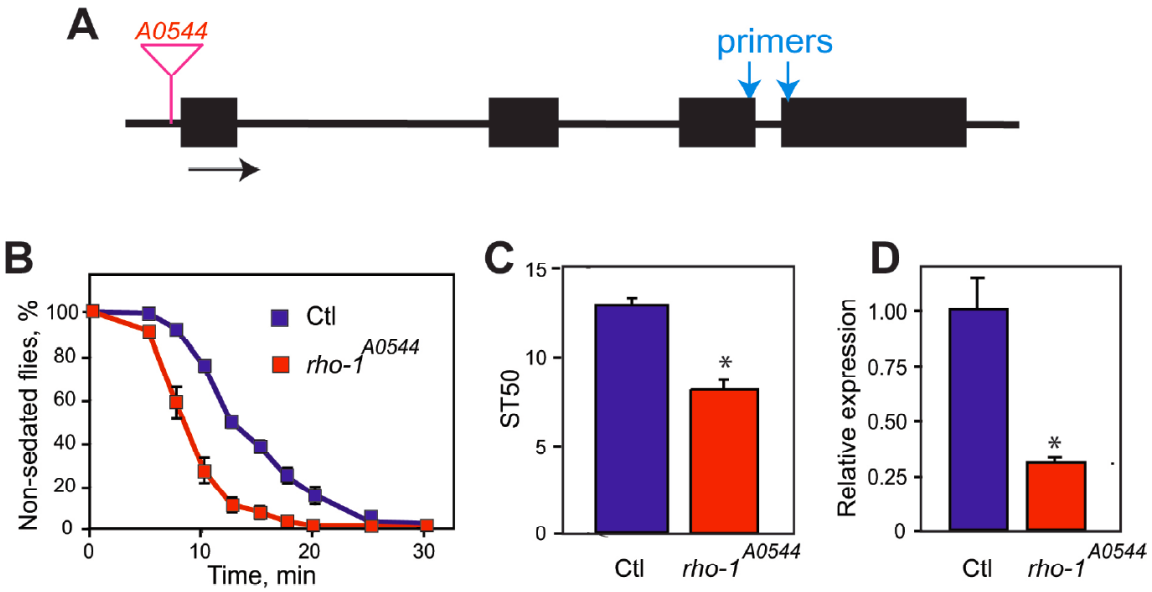
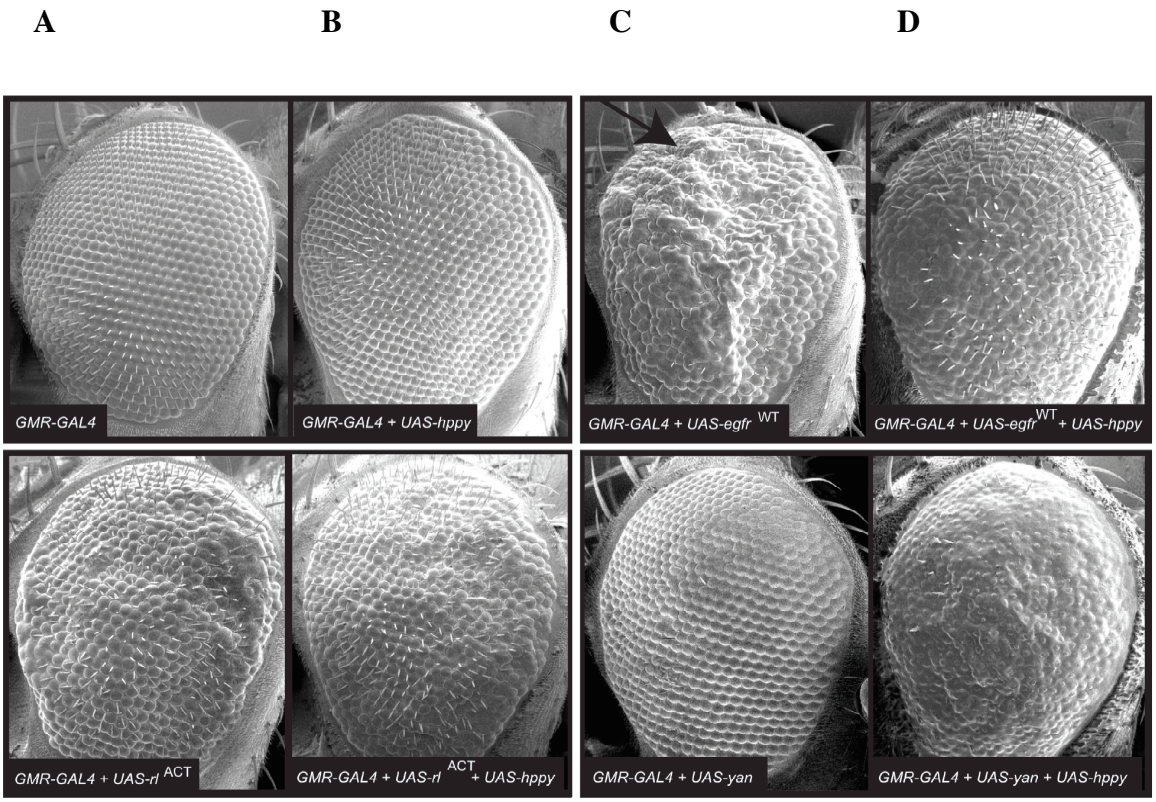
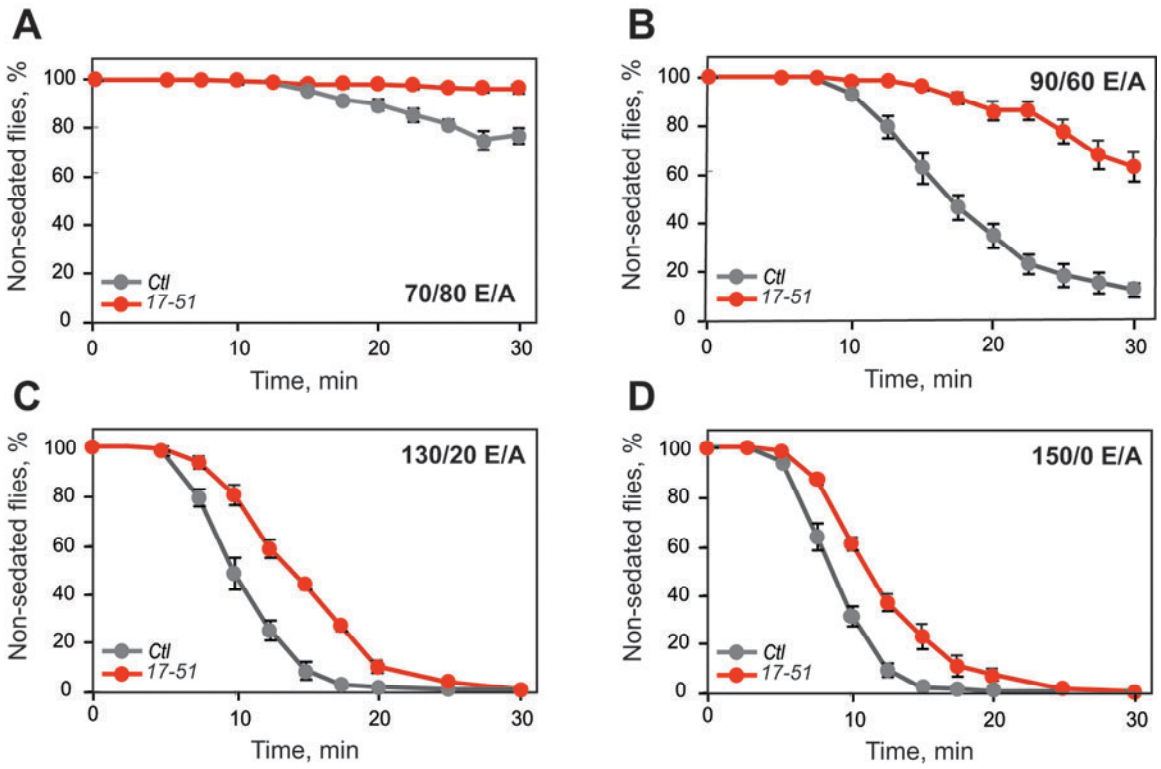

A
A0544
primers
B
Non-sedated flies, %
100
80
60
40
20
0
0
10
20
30
Time, min
Ctl
rho-1A0544
C
ST50
15
10
5
0
*
Ctl
rho-1A0544
D
Relative expression
1.00
0.75
0.50
0.25
0
*
Ctl
rho-1A0544

Figure 7


A
B
C
D
GMR-GAL4
GMR-GAL4 + UAS-hppy
GMR-GAL4 + UAS-egfr WT
GMR-GAL4 + UAS-egfr WT + UAS-hppy
GMR-GAL4 + UAS-rl ACT
GMR-GAL4 + UAS-rl ACT + UAS-hppy
GMR-GAL4 + UAS-yan
GMR-GAL4 + UAS-yan + UAS-hppy
E
F
G
H

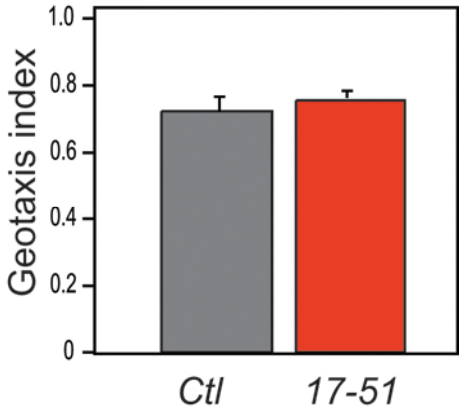
Supplementary Information

Supplementary Figure 1. *hppy*$^{17\text{-}51}$ flies are resistant to sedation when exposed to a broad range of ethanol concentrations.



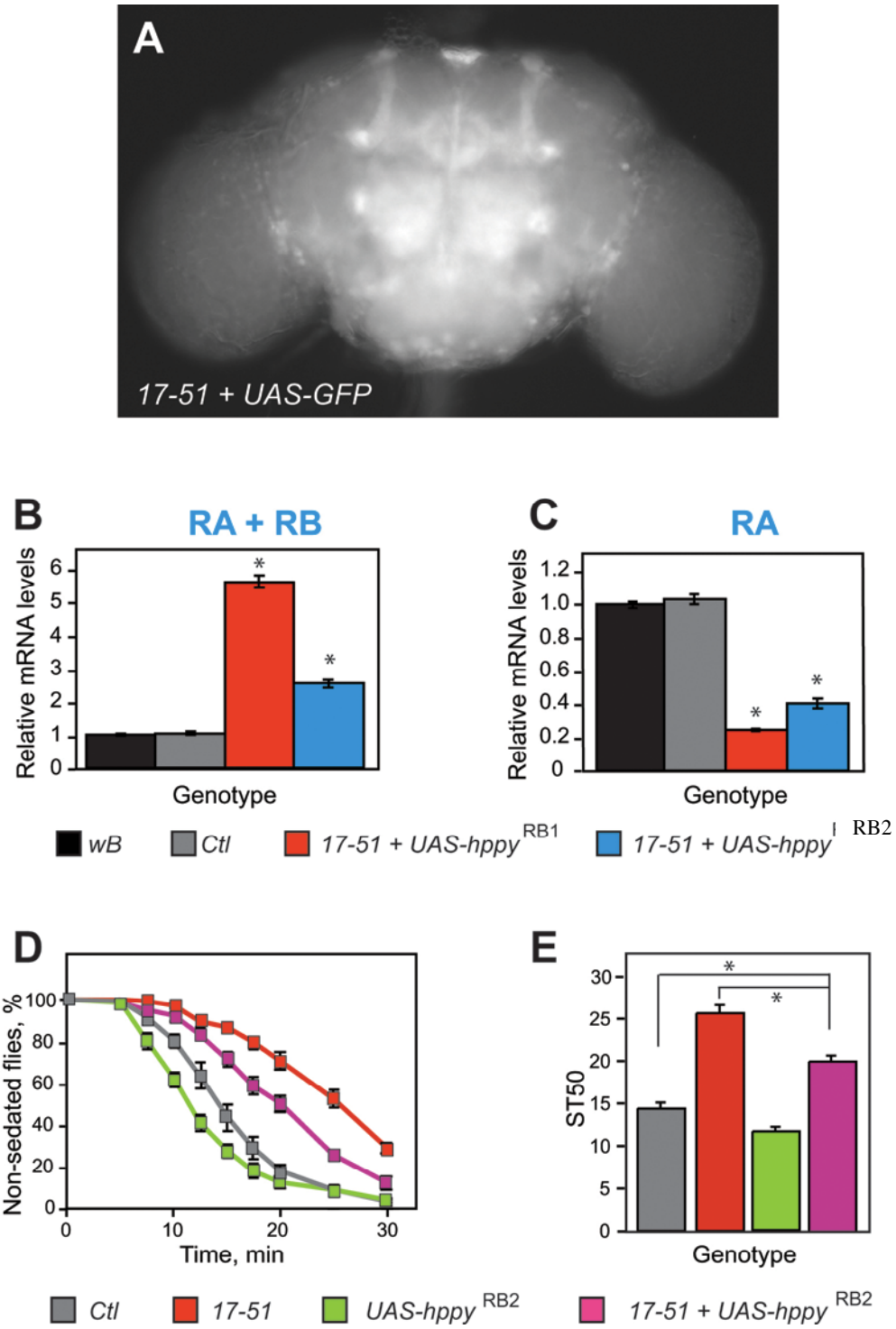
Sedation profiles were calculated for *hppy*$^{17\text{-}51}$ flies and the control line 8-165 (*Ctl*) flies at a variety of ethanol concentrations. Ethanol exposure commenced at 0 min and was continuous thereafter. Ethanol/air (E/A) concentrations tested were: (**A**) 70/80 E/A, (**B**) 90/60 E/A, (**C**) 130/20 E/A, and (**D**) 150/0 E/A. n=8.

Supplementary Figure 2. *hppy*$^{17\text{-}51}$ flies do not have defects in negative geotaxis.



The ability of *hppy*$^{17\text{-}51}$ and control (line 8-165) flies to climb to the top of a glass cylinder after being banged down to the bottom was measured (see Methods). Student's paired t-test assuming equal variances failed to reveal a significant difference between genotypes (p=0.4852). n=25.

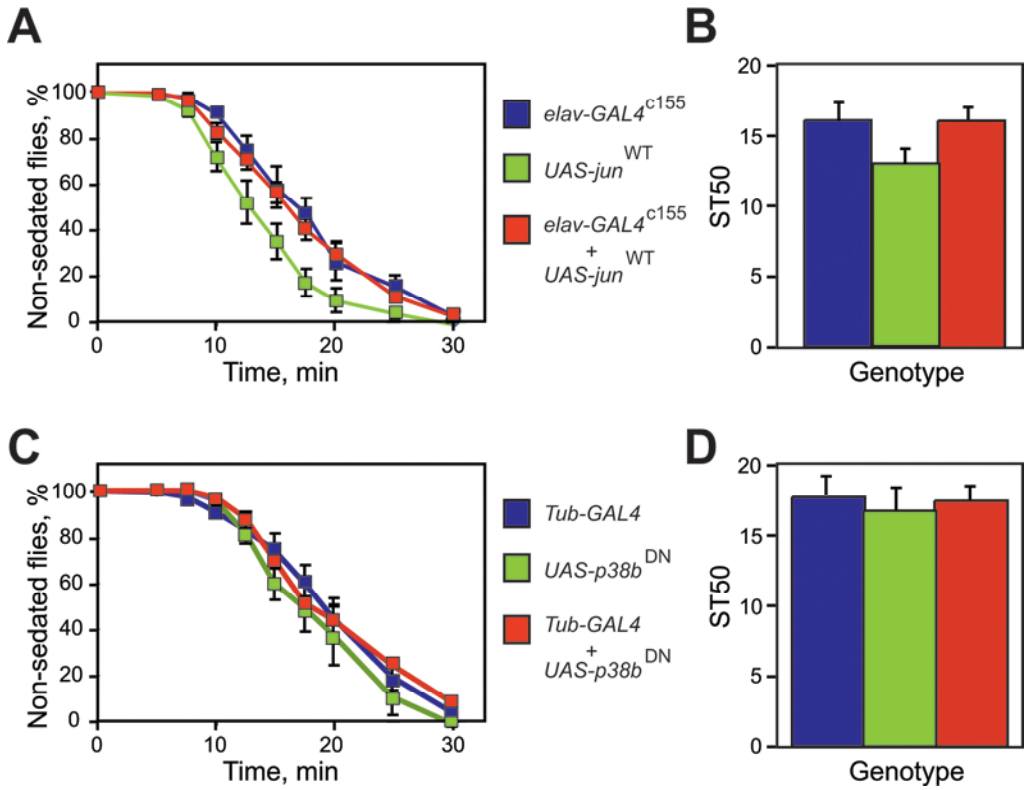
Supplementary Figure 3. Expression of *hppy*$^{17\text{-}51}$ and rescue by *UAS-hppy*^{RB2} transgene.



(**A**) The *hppy*$^{17\text{-}51}$ GAL4 expression pattern is widespread in the fly CNS. Pictured is a whole mount adult brain of a fly carrying *hppy*$^{17\text{-}51}$ and *UAS-GFP*. (**B**) Quantitative RT-PCR on whole flies using a primer/probe set recognizing both *hppy-RA* and *hppy-RB* transcripts shows that *UAS-hppy* expression is increased in *hppy*$^{17\text{-}51}$*;UAS-hppy*^{RB} flies. *UAS-hppy*^{RB1} and *UAS-hppy*^{RB2} represent two different insertions lines of the *UAS-hppy*^{RB} transgene. All behavioral experiments presented in the main body of the paper utilized the more strongly expressing, *UAS-hppy*^{RB1} insertion line. One-way ANOVA revealed a significant difference between genotypes (p<0.001). Post-hoc Newman-Keuls analysis revealed a significant difference between *hppy*$^{17\text{-}51}$*; UAS-hppy*^{RB1} and both *w* Berlin (p<0.001) and Control (line 8-165) (p<0.001) as well as a significant difference between *hppy*$^{17\text{-}51}$*;UAS-hppy*^{RB2} and both *w* Berlin (p<0.001) and Control (p<0.001). n=3. (**C**) Quantitative RT-PCR on whole flies using a primer/probe set recognizing specifically the *hppy-RA* transcript shows that the *hppy-RA* transcript is not increased in *hppy*$^{17\text{-}51}$*;UAS-hppy*^{RB} flies. *UAS-hppy*^{RB1} and *UAS-hppy*^{RB2} represent two different insertions lines of the *UAS-hppy*^{RB} transgene. All behavioral experiments presented in the main body of the paper utilized the more strongly expressing, *UAS-hppy*^{RB1} insertion line. One-way ANOVA revealed a significant difference between genotypes (p<0.001). Post-hoc Newman-Keuls analysis revealed a significant difference between *hppy*$^{17\text{-}51}$*;UAS-hppy*^{RB1} and both *w* Berlin (p<0.001) and Control line 8-165 (p<0.001) as well as a significant difference between *hppy*$^{17\text{-}51}$*;UAS-hppy*^{RB2} and both *w* Berlin (p<0.001) and Control (p<0.001). n=3. (**D, E**) The *hppy*$^{17\text{-}51}$ sedation resistance phenotype can be partially rescued by expression of the *UAS-hppy*^{RB2} transgene in the *hppy*$^{17\text{-}51}$ mutant background. (**D**) Sedation profiles and (**E**) ST50 values are shown. One-way ANOVA of the ST50

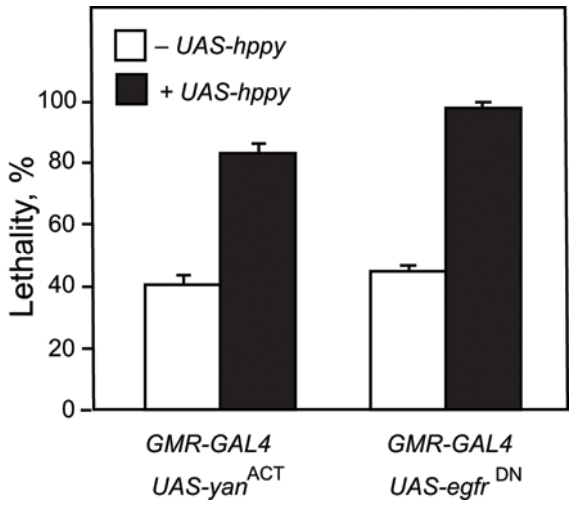
values revealed a significant difference between genotypes (p<0.0001). Post-hoc Newman-Keuls analysis revealed a significant difference between *hppy*$^{17\text{-}51}$*;UAS-hppy*^{RB2} and *hppy*$^{17\text{-}51}$ (p<0.001). *hppy*$^{17\text{-}51}$*;UAS-hppy*^{RB2} was also significantly different from Control (p<0.001) and *UAS-hppy*^{RB2} (p<0.001). n=8.

Supplementary Figure 4. Manipulations of the JNK and p38 pathways do not affect ethanol-induced sedation.



(**A, B**) Flies expressing the wild-type transcription factor dJun, *UAS-Jun*^{WT}, under the control of the pan-neuronal driver *elav-GAL4*^{c155} have an ST50 similar to those of controls. (**A**) Sedation profiles and (**B**) ST50 values are shown. One-way ANOVA of the ST50 values failed to reveal a significant difference between genotypes (p=0.0611). n=8. (**C, D**) Flies expressing a dominant negative form of the MAPK p38β, *UAS-p38b*^{DN}, under the control of the pan-organismal driver *Tub-GAL4* have an ST50 similar to those of controls. (**C**) Sedation profiles and (**D**) ST50 values are shown. One-way ANOVA of the ST50 values failed to reveal a significant difference between genotypes (p=0.8513). n=4.

Supplementary Figure 5. Interactions between *hppy* and EGFR pathway components on fly viability.



Expression of *hppy-RB* enhances the semi-lethality induced by *GMR-GAL4* driven expression of a dominant negative form of the EGFR, *UAS-egfr*^{DN}, as well as expression of an activated form of the EGFR/ERK pathway inhibitor Yan, *UAS-yan*^{ACT}. Adult flies were counted five days after eclosion and % lethality was calculated as described (see Supplementary Methods). Student's paired *t*-test assuming equal variance revealed a significant difference between *GMR-GAL4;UAS-egfr*^{DN} and *GMR-GAL4;UAS-hppy*^{RB1}*;UAS-egfr*^{DN} (p=0.0027) as well as between *GMR-GAL4;UAS-yan*^{ACT} and *GMR-GAL4;UAS-hppy*^{RB1}*;UAS-yan*^{ACT} (p=0.0064). Each experiment was repeated on three different days (n=3).

Supplementary Methods

Negative Geotaxis Assay The negative geotaxis assay, which also measures locomotion and responsiveness to banging, was performed as described previously (Moore et al., 1998) with the following exceptions. The dimensions for the cylinder used were 22.5 cm in length by 2.7 cm in width. After loading the ten 2-4 day old flies of each genotype into a cylinder, the flies were banged down to the bottom and observed as they climbed to the top. At the end of each one minute period, the number of flies which had reached the top of the cylinder were counted and the flies were banged down once more. This process was repeated at 1 minute intervals for 5 minutes.

Lethality Enhancement Assay

Five by five crosses in vials were set up and raised at 25°C. For these crosses, *GMR-GAL4* or *GMR-GAL4; UAS-hppy*^{RB1} virgins were crossed to *UAS-yan*^{ACT}*/CyO* or *UAS-egfr*^{DN}*/CyO* males. Parents were cleared on day 2 and progeny were scored for the presence or absence of the CyO balancer chromosome on day 15. By Mendelian genetics, the number of progeny bearing the CyO balancer chromosome should make up 50% of the progeny in each cross. Percent lethality for each of these four genotypes: [1.) *GMR-GAL4, UAS-yan*^{ACT} 2.) *GMR-GAL4, UAS-yan*^{ACT}*; UAS-hppy*^{RB1} 3.) *GMR-GAL4, UAS-egfr*^{DN} 4.) *GMR-GAL4, UAS-egfr*^{DN}*; UAS-hppy*^{RB1}*]* was calculated using the following equation: % lethality = (1 – (# of non-Cy winged progeny / # of Cy winged progeny)) * 100%

Light Microscopy

To visualize the 17-51 GAL4 expression pattern, 17-51 virgins were crossed to males of a line carrying the GFP reporter gene under the control of a UAS enhancer element (UAS-GFP T2). After dissecting out the brains of 2-4 day old adult male progeny in 1xPBS, specimens were washed in 1xPBS and visualized using a Zeiss Axioskop II microscope.

Chapter 4

Insulin signaling in the nervous system regulates ethanol intoxication in *Drosophila melanogaster*

Ammon B. Corl, Aylin R. Rodan, and Ulrike Heberlein

This work was published previously as:

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Abstract

The insulin signaling pathway regulates multiple physiological processes, including energy metabolism, organismal growth, aging and reproduction. Here we show that genetic manipulations in *Drosophila melanogaster* that impair the function of insulin-producing cells or of the insulin-receptor signaling pathway in the nervous system lead to increased sensitivity to the intoxicating effects of ethanol. These findings suggest a previously unknown role for this highly conserved pathway in regulating the behavioral responses to an addictive drug.

Introduction

Although ethanol is one of the most widely abused drugs in the world, the mechanisms that mediate its behavioral effects are still poorly understood. *Drosophila melanogaster*, with its accessibility to genetic, molecular, and behavioral analyses, has been developed as a promising model system in which to identify conserved genes and pathways that regulate ethanol-induced behaviors (Wolf and Heberlein, 2003). For example, studies have implicated the cAMP signal-transduction pathway in the regulation of acute ethanol sensitivity in flies and mice (Moore et al., 1998; Park et al., 2000; Thiele et al., 2000). In flies, a forward genetic screen to identify *Drosophila* mutants with altered sensitivity to the intoxicating effects of ethanol led to the isolation of the gene *amnesiac*, which codes for a putative neuropeptide that is thought to activate cAMP signaling (Moore et al., 1998; Feany and Quinn, 1995). Subsequent experiments demonstrated that inhibition of the cAMP-dependent protein kinase (PKA) pathway through genetic or pharmacological methods also resulted in increased sensitivity to ethanol (Moore et al.,

1998). Tissue specific expression of an inhibitor of PKA through the use of the GAL4/UAS binary expression system (Brand and Perrimon, 1993) allowed us to identify specific subsets of the *Drosophila* central nervous system that are involved in regulating ethanol sensitivity (Rodan et al., 2002). Through these studies, a requirement for proper cAMP signaling was been mapped to a small group of neurosecretory cells located in the dorsal/medial region of the adult brain (Rodan et al., 2002). Clusters of cells producing insulin-like peptides (DILPs) are found in an equivalent location in the brain (Brogiolo et al., 2001; Rulifson et al., 2002) and therefore have become attractive candidate cells for regulating ethanol sensitivity.

Results and Discussion

To determine whether insulin-producing cells (IPCs) regulate ethanol sensitivity, we used the GAL4-UAS binary expression system (Brand and Perrimon, 1993) to express an inhibitor of cAMP-dependent protein kinase (*UAS-PKA*^{inh}) (Rodan et al., 2002) selectively in these cells. When GAL4 is placed under the regulation of the *dilp2* promoter in *dilp2-GAL4*, the transgene drives expression of GAL4 specifically in the IPCs of the developing (Rulifson et al., 2002) and adult brain (Fig. 1a). The ethanol sensitivity of flies carrying both *dilp2-GAL4* and *UAS-PKA*^{inh} was tested by introducing the flies into the inebriometer, a 4 foot tall baffle filled column through which ethanol vapors are circulated (Cohan and Hoffman, 1986; Weber 1988). As flies become intoxicated, they tumble, or elute, out of the bottom of the inebriometer and are counted in three-minute bins. A mean elution time (MET) can then be calculated for each genotype. *Dilp2-GAL4 + UAS-PKA*^{inh} flies showed significantly increased ethanol

sensitivity, manifested as a reduced mean elution time in the inebriometer, compared to control strains (Fig. 1b). This effect is specific to inhibition of protein kinase A (PKA), as it was not observed when an inactive PKA inhibitor (*UAS-PKA*$^{m\text{-}inh}$) was expressed in the IPCs (Fig. 1b). PKA inhibition in the *dilp2-GAL4* cells does not kill the IPCs, as X-Gal staining in *dilp2-GAL4 + UAS-PKA*^{inh} flies carrying *dilp2-lacZ*, which drives β-galactosidase expression in the IPCs (Ikeya et al., 2002), was indistinguishable from that of controls (Fig. 1c and Fig. 1d).

We postulated that inhibition of PKA in IPCs leads to reduced DILP production and/or secretion, which in turn causes increased ethanol sensitivity. If this is correct, then reduced function of the insulin receptor (InR) or of its signaling pathway should result in a similar ethanol-sensitivity defect. We therefore tested flies heterozygous for mutations in the InR (alleles *InR*^{GC25} and *InR*^{E19}, see Methods), which show 25-50% reductions in InR kinase activity (Chen et al., 1996; Tatar et al., 2001). These flies, although healthy and of normal size, showed increased ethanol sensitivity in the inebriometer (Fig. 2a). We also tested flies heterozygous for a null mutation in the gene encoding the insulin-receptor substrate CHICO (Bohni et al., 1999); these flies were similarly sensitive in the inebriometer (Fig. 2a). In addition, flies bearing a P-element insertion in the gene coding for the *Drosophila* homologue of phosphatidylinositol-dependent-kinase-1 (dPDK-1 or Dstpk61), a downstream target of insulin signaling, also showed increased sensitivity in the inebriometer (Fig. 2b). Thus, global perturbations in insulin signaling increase ethanol sensitivity.

DILPs could regulate ethanol sensitivity by acting directly in the nervous system or by regulating a secondary signal made elsewhere. To distinguish these possibilities, we

expressed in the nervous system, using the pan-neuronal *elav-GAL4*^{3E1} driver, molecules known to inhibit InR signaling. First, we expressed an inhibitor of phosphatidylinositol 3-kinase (PI3K), UAS-P60, which is believed to interfere with the recruitment of endogenous PI3K upon InR activation (Weinkove et al., 1999). Flies carrying UAS-P60 and the neuronal driver *elav-GAL4*^{3E1} showed increased ethanol sensitivity compared to control flies (Fig. 3a). Second, we tested the effects of neuronal expression of the forkhead-related (FKHR) transcription factor hFOXO3a, which is negatively regulated by insulin signaling through phosphorylation by protein kinase B (Junger et al., 2003). Expression of a constitutively active form of hFOXO3a, *UAS-hFOXO3a-TM*, whose function is not inhibited upon activation of the InR pathway and which thus acts as a constitutive blocker of the InR pathway (Junger et al., 2003), also caused increased ethanol sensitivity (Fig. 3a). Notably, this effect was not observed upon expression of the wild-type hFOXO3a gene, *UAS-hFOXO3a* (Fig. 3a). Finally, we found that neuronal expression of an RNA interference construct targeted against the insulin receptor, *UAS-InR*^{RNAi}, also resulted in increased ethanol sensitivity in the inebriometer (Fig. 3b). Taken together, these data show that inhibition of the InR pathway specifically in the nervous system causes increased sensitivity to the intoxicating effects of ethanol.

The behavioral changes observed were brought about by subtle changes in InR signaling. Although strong or complete inhibition of the pathway causes severe developmental delay, a reduction in body size, and even death, all flies tested here developed normally, had wild-type (or nearly wild-type) body size, and performed normally in several assays that measure baseline climbing and walking behavior. All genotypes also showed normal ethanol absorption (Fig. 4).

In summary, we show that normal function of the IPCs and of the InR pathway are necessary for *Drosophila melanogaster* to show normal sensitivity to the acute intoxicating effects of ethanol. In mammalian models, neurons of the mesolimbic dopamine system, which regulate acute drug sensitivity and the rewarding properties of drugs and food, have been shown to be targets of insulin action (Figlewicz, 2003). This suggests that insulin has an evolutionarily conserved role in regulating the responsiveness of the nervous system to intoxicating drugs.

Materials and Methods

Fly stocks and genetics

Flies carrying *dilp2-GAL4* were obtained from E. Rulifson (Rulifson et al., 2002), *UAS-PKA*^{inh} flies (also called BDK33) from D. Kalderon (Li et al., 1995), *UAS-PKA*$^{m\text{-}inh}$ flies from J. Kiger (Kiger and O'Shea, 2001). *InR*^{E19}, *InR*^{GC25}, and *chico*^{1} mutants outcrossed to the Dahomey genetic background, as well as the Dahomey background control flies, were obtained from D. Gems (Clancy et al., 2001), *elav-GAL4*^{3E1} flies were obtained from S. Sweeney (Davis et al., 1997), *UAS-p60* flies from E. Rulifson (Weinkove et al., 1999), *UAS-hFOXO3a* flies and *UAS-hFOXO3a-TM* flies from E. Hafen (Junger et al., 2003). *Dilp2-lacZ* flies were obtained from E. Hafen (Ikeya et al., 2002). EP(3)3644, bearing a P-element insertion in the *dPDK-1* gene region, and control line EP(3)1015 were obtained from F. Wolf, UCSF. All lines used for behavioral experiments (with the exception of *InR* and *chico* mutants) were outcrossed for five generations to a w^{1118} stock isogenic for chromosomes II and III.

The *UAS-InR-RNAi* transgene was created by M. Moore and J. Gesch, UCSF. Primer sequences used to amplify the fragment of the *InR* gene region to be inserted into the *UAS-InR-RNAi* construct were: Forward: 5' TTTGCACGAGACTGACAGATAC 3' Reverse: 5' GGTATAACTTTACACCGGCCAAC 3'.

For behavioral testing, flies carrying both GAL4 and UAS insertions were generated by crossing GAL4 virgin females to UAS males. As controls, GAL4 or UAS heterozygotes were generated by crossing GAL4 virgins to w^{1118} males or by crossing w^{1118} virgins to UAS-males. *InR* and *chico*^{1} mutants as well as Dahomey background control flies were tested as heterozygotes by crossing males to virgin females carrying attached X chromosomes in the w^{1118} genetic background and selecting against balancers in the subsequent generation.

Flies were raised on standard cornmeal and molasses food at 25°C and 70% relative humidity. All experiments were performed on 2-5 day old males at 20°C, utilizing ~110 males for each inebriometer run. All genotypes were tested across multiple days.

Inebriometer assay

Flies were tested in the inebriometer as described previously (Moore et al., 1998). Inebriometers were set to an ethanol/humidified air mixture of ~60/35 E/A and were allowed to equilibrate to 20°C. Flies were allowed to equilibrate for 5 minutes at 20°C before being introduced into the inebriometer. As flies eluted from the inebriometer, they

were counted in 3-minute bins by a *Drosophila* activity monitor (Trikinetics, Waltham, MA). Mean elution times (METs) were then calculated from the resulting elution profiles. Inebriometer phenotypes of various lines were only considered different from controls if they were found to be significantly different from all applicable controls.

Ethanol absorption

Twenty-five flies of each genotype were exposed in triplicate to an ethanol/humidified air mixture of 50/100 E/A for 0, 10, 15, 20, or 30 minutes in perforated test tubes. Following exposure to ethanol, flies were frozen in dry ice and homogenized in 500 μL of 50 mM Tris-HCl (pH 7.5). Ethanol assays were then performed on the fly homogenates as previously described (Moore et al., 1998).

Histochemistry

Confocal microscopy

To determine the adult CNS *dilp2-GAL4* expression pattern, *dilp2-GAL4* virgins were crossed to *UAS-GFP T2; UAS-Tau GFP* males (double transgenic stock created by F. Wolf, UCSF). Brains and ventral nerve cords were dissected from 2-4 day old adult male progeny in 1xPBS and fixed in 4% paraformaldehyde for 20 minutes. After washing in 1x PBS, neuropil labeling was achieved by incubating specimens in a 1:50 dilution of Nc82 antibody obtained from S. Sweeney, UCSF (Jefferis et al., 2001) and with a Cy3 coupled goat anti-mouse antibody, diluted 1:500 (Molecular Probes, Eugene, OR). Specimens were mounted in Vectashield mounting medium (Vector laboratories,

Burlingame, CA) and analyzed with a Leica confocal microscope with Leica Confocal Software Version 2.5.

Light Microscopy

For experiments visualizing lacZ staining in *dilp* neurosecretory cells, virgins bearing both the *dilp2-GAL4* and *dilp2-lacZ* transgenes were crossed to males bearing either the *UAS-PKA*^{inh} or the *UAS-PKA*$^{m\text{-}inh}$ transgene. After dissecting out the brains of 2-4 day old adult male progeny, X-Gal staining was performed as previously described (Rodan et al., 2002) and specimens were visualized using a Zeiss Axioskop II microscope.

Statistics

Data from multiple genotypes were compared using either Student's unpaired t-tests assuming equal variance or one-way ANOVA tests followed by post-hoc Newman-Keuls testing using GraphPad Prism software, Version 4 (Graphpad, San Diego, CA). Error bars in all experiments represent the standard error of the mean (s.e.m.).

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Figure Legends

Figure 1: PKA inhibition in IPCs results in increased ethanol sensitivity.

(A) *dilp2-GAL4* drives expression specifically in the IPCs of the adult *D. melanogaster* CNS. Pictured is a confocal microscope image of the adult brain (top) and ventral nerve cord (VNC, bottom) of a fly carrying *dilp2-GAL4* and *UAS-GFP*. Expression of GFP is green and expression of the general neuropil marker Nc82 is purple. OL, optic lobes; ES, esophagus.

(B) Flies carrying the *UAS-PKA*^{inh} and *dilp2-GAL4* transgenes show a significantly reduced MET in the inebriometer compared to either transgenic line alone. One-way ANOVA revealed a significant difference between genotypes ($p<0.0001$). Post-hoc Newman-Keuls analysis revealed a significant difference between *dilp2-GAL4+UAS-PKA*^{inh} and *dilp2-GAL4* ($p<0.001$) as well as between *dilp2-GAL4+UAS-PKA*^{inh} and *UAS-PKA*^{inh} ($p<0.001$). n=6. The MET of flies carrying the inactive inhibitor *UAS-PKA*$^{m\text{-}inh}$ and *dilp2-GAL4* was not significantly different from that of controls. One-way ANOVA revealed a significant difference between genotypes ($p=0.0025$). While post-hoc Newman-Keuls analysis did reveal a significant difference between *dilp2-GAL4+UAS-PKA*$^{m\text{-}inh}$ and *UAS-PKA*$^{m\text{-}inh}$ ($p<0.01$), it failed to reveal a difference between *dilp2-GAL4+UAS-PKA*$^{m\text{-}inh}$ and *dilp2-GAL4* ($p>0.05$). n=4-6. In this and all figures, stars denote significant differences and error bars indicate standard error of the mean (SEM).

(C) and (D) PKA inhibition in *dilp2-GAL4* cells does not appear to kill the IPCs. X-gal staining of the IPCs in *dilp2-GAL4+UAS-PKA*^{inh}+*dilp2-lacZ* flies (Figure 1c.) looks comparable to X-gal staining of the IPCs in control (*dilp2-GAL4+UAS-PKA*$^{m\text{-}inh}$+*dilp2-lacZ*) flies (Figure 1d.). Images were taken at 1000x magnification after one hour of X-gal staining.

Figure 2: Mutations in the components of the insulin signaling pathway cause increased ethanol sensitivity.

(A) Mutations in the InR and CHICO cause increased ethanol sensitivity. *InR*^{E19}, *InR*^{GC25}, and *chico*^{1} were tested in heterozygous form because of the low viability and severe developmental delay of homozygous flies. All mutant flies showed a significant reduction in MET compared to their appropriate genetic controls (+/+). One-way ANOVA revealed a significant difference between genotypes ($p=0.0007$). Post-hoc Newman-Keuls analysis revealed a significant difference between +/+ and *InR*^{E19}/+ ($p<0.01$), between +/+ and *InR*^{GC25}/+ ($p<0.001$), as well as between +/+ and *chico*^{1} ($p<0.001$). n=4.

(B) EP(3)3644 flies, carrying a P-element insertion in the *dPDK-1* (*Dstpk61*) gene, have increased ethanol sensitivity. Student's unpaired t-test assuming equal variance revealed a significant difference between EP(3)3644 and control (EP(3)1015) flies ($p<0.0001$). n=4.

Figure 3: Perturbation of insulin signaling in the nervous system alters ethanol sensitivity

(A) Left panel: Flies expressing the PI3K inhibitor UAS-P60 in neurons under the control of the *elav-GAL4*^{3E1} driver show a significantly reduced MET compared to singly transgenic controls. One-way ANOVA revealed a significant difference between genotypes ($p<0.0001$). Post-hoc Newman-Keuls analysis revealed a significant difference between *elav-GAL4*^{3E1}+*UAS-P60* and *elav-GAL4*^{3E1} ($p<0.001$) as well as between *elav-GAL4*^{3E1} +*UAS-P60* and *UAS-P60* ($p<0.001$). n=8-10.

(A) Right panel: Neuronal expression of a "superactive" hFOXO3a, *UAS-hFOXO3a-TM*, under the control of the *elav-GAL4*^{3E1} driver results in increased ethanol sensitivity. One-way ANOVA revealed a significant difference between genotypes ($p<0.0001$). Post-hoc Newman-Keuls analysis revealed a significant difference between *elav-GAL4*^{3E1}+*UAS-hFOXO3a-TM* and *elav-GAL4*^{3E1} ($p<0.01$) as well as between *elav-GAL4*^{3E1}+*UAS-hFOXO3a-TM* and *UAS-hFOXO3a-TM* ($p<0.001$). n=5-6. Neuronal expression of wild-type hFOXO3a, *UAS-hFOXO3a*, had no effect. One-way ANOVA revealed a significant difference between genotypes ($p=0.0311$). However, post-hoc Newman-Keuls analysis failed to reveal a significant difference between *elav-GAL4*^{3E1}+*UAS-hFOXO3a* and *elav-GAL4*^{3E1} ($p>0.05$) and also failed to reveal a significant difference between *elav-GAL4*^{3E1}+*UAS-hFOXO3a* and *UAS-hFOXO3a* ($p>0.05$). n=5.

(B) Neuronal expression of three independent inserts of an RNAi construct directed against InR, *UAS-InR-RNAi*, results in increased ethanol sensitivity. One-way ANOVA

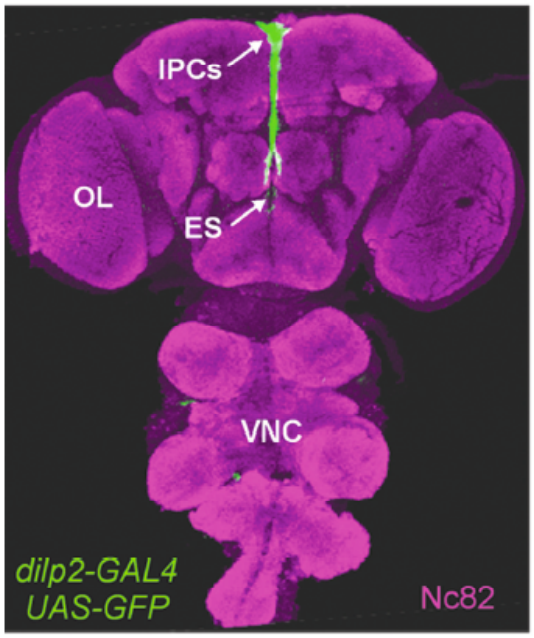
between *elav-GAL4*^{3E1}+*UAS-InR-RNAi-a* flies versus singly transgenic controls revealed a significant difference between genotypes (p=0.0003). Post-hoc Newman-Keuls analysis revealed a significant difference between *elav-GAL4*^{3E1}+*UAS-InR-RNAi-a* and *elav-GAL4*^{3E1} (p<0.001) as well as between *elav-GAL4*^{3E1}+*UAS-InR-RNAi-a* and *UAS-InR-RNAi-a* (p<0.001). n=4. One-way ANOVA between *elav-GAL4*^{3E1}+*UAS-InR-RNAi-b* flies versus singly transgenic controls revealed a significant difference between genotypes (p=0.0109). Post-hoc Newman-Keuls analysis revealed a significant difference between *elav-GAL4*^{3E1}+*UAS-InR-RNAi-b* and *elav-GAL4*^{3E1} (p<0.05) as well as between *elav-GAL4*^{3E1}+*UAS-InR-RNAi-b* and *UAS-InR-RNAi-b* (p<0.05). n=4. One-way ANOVA between *elav-GAL4*^{3E1}+*UAS-InR-RNAi-c* flies versus singly transgenic controls revealed a significant difference between genotypes (p=0.0142). Post-hoc Newman-Keuls analysis revealed a significant difference between *elav-GAL4*^{3E1}+*UAS-InR-RNAi-c* and *elav-GAL4*^{3E1} (p<0.05) as well as between *elav-GAL4*^{3E1}+*UAS-InR-RNAi-c* and *UAS-InR-RNAi-c* (p<0.05). n=4.

Figure 4: Inhibition of PKA in IPCs does not cause alterations in ethanol pharmacokinetics.

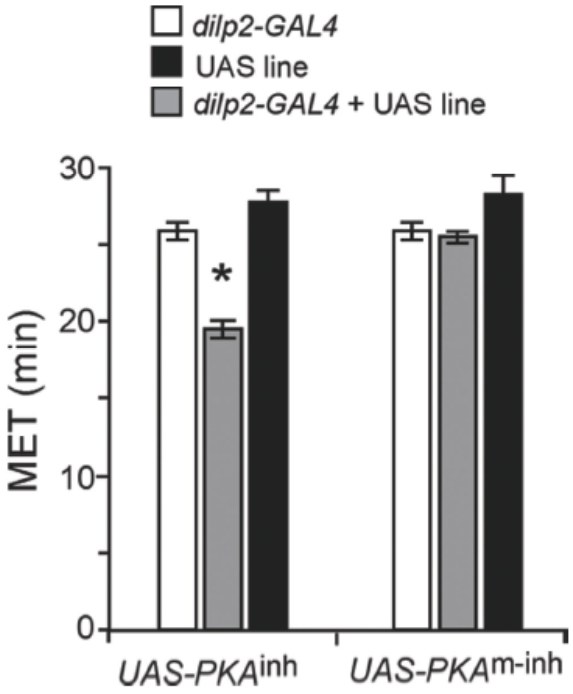
Ethanol levels were measured in extracts of flies of the indicated genotypes (see Methods for details). Ethanol exposure is continuous starting at time = 0. One-way ANOVA across each time point, with the critical p value adjusted to $\alpha = 0.01$, did not reveal significant differences between the genotypes at any of the five time points (p>0.05). n=3 for all genotypes.

Figure 1

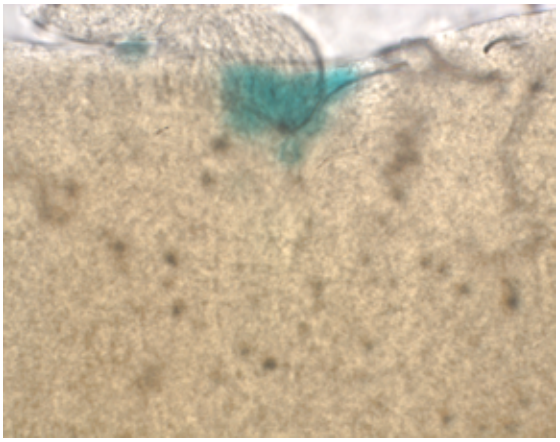
A



B



C



D

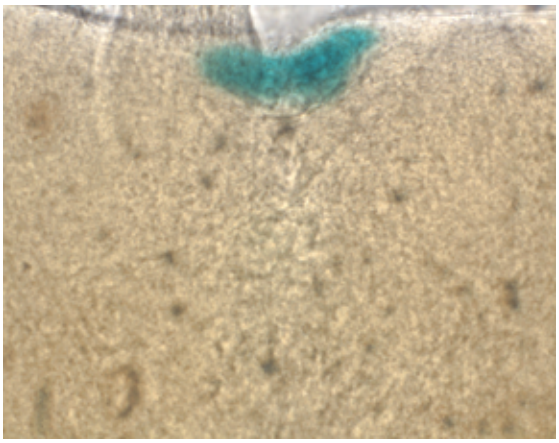
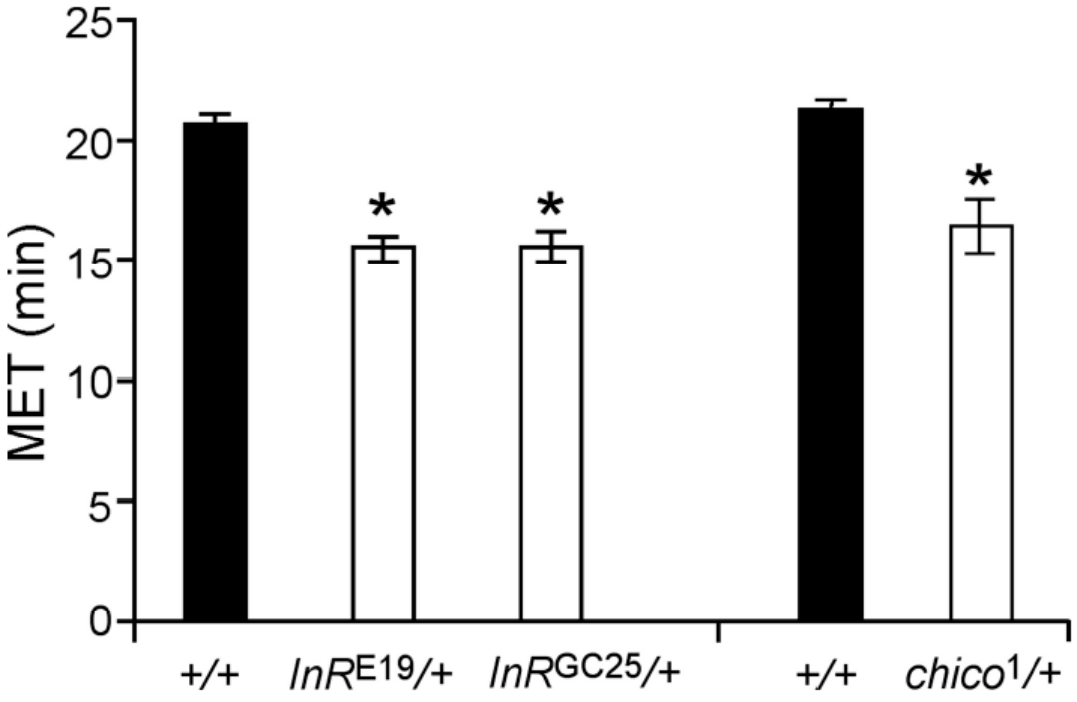


Figure 2

A


25
20
15
10
5
0
MET (min)
*
*
*
+/+
InR^E19/+
InR^GC25/+
+/+
chico^1/+

B

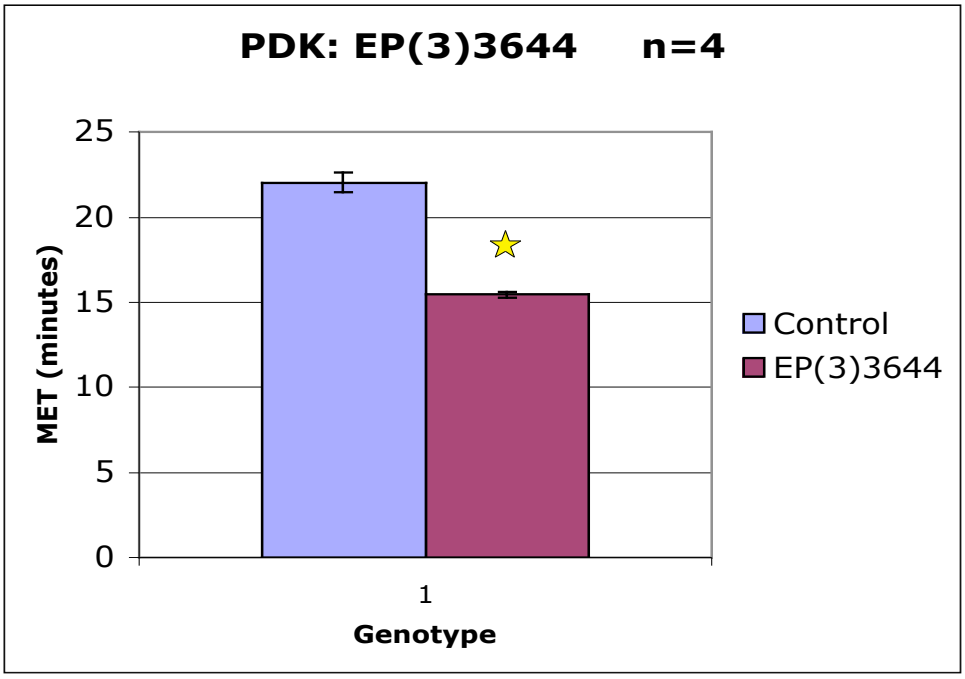
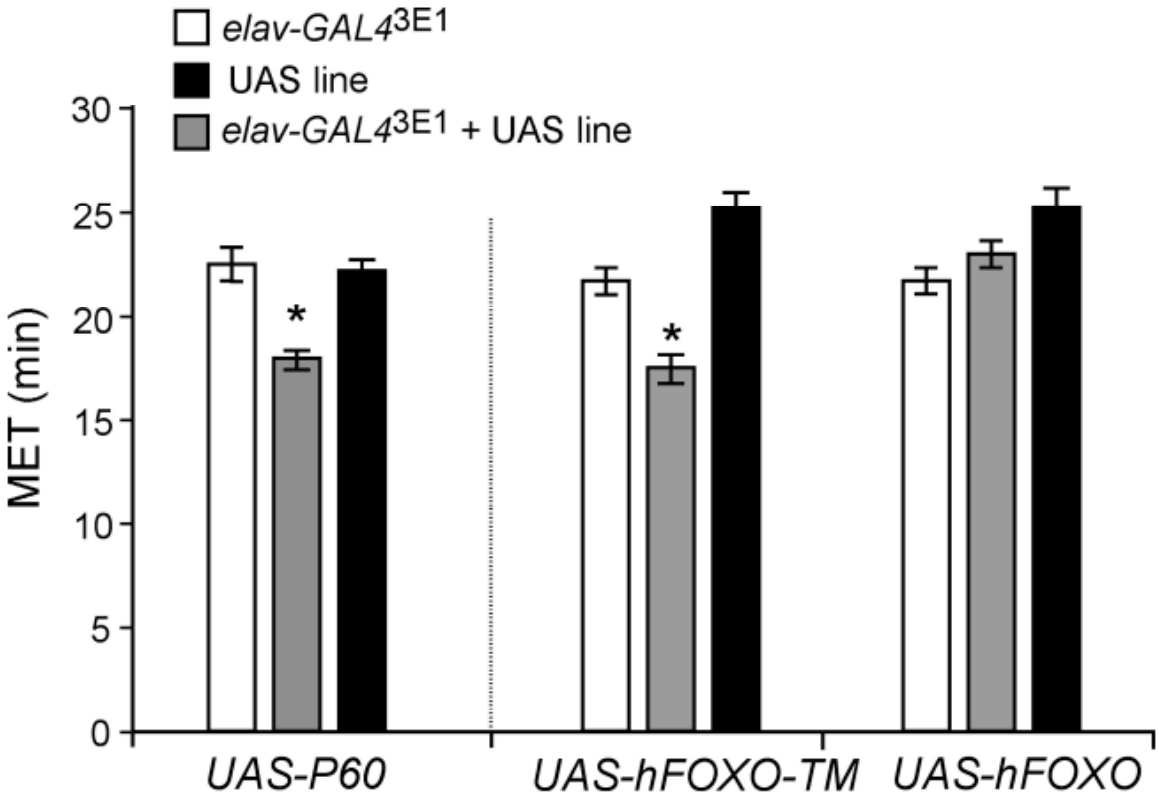

PDK: EP(3)3644 n=4
25
20
15
10
5
0
MET (minutes)
1
Genotype
Control
EP(3)3644

Figure 3

A


elav-GAL4 3E1
UAS line
elav-GAL4 3E1 + UAS line
MET (min)
30
25
20
15
10
5
0
*
*
UAS-P60
UAS-hFOXO-TM
UAS-hFOXO

B

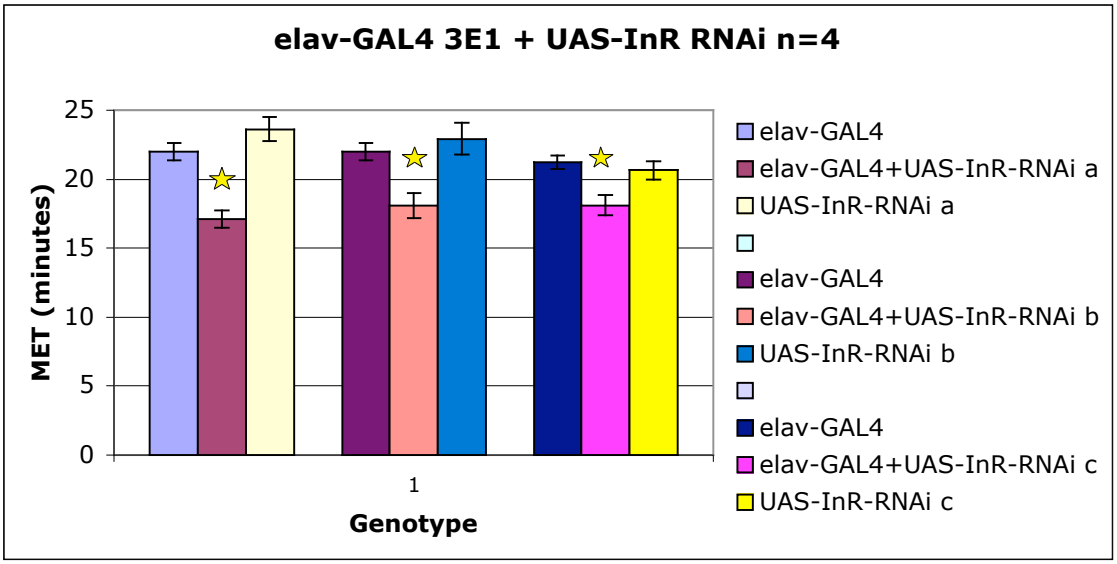
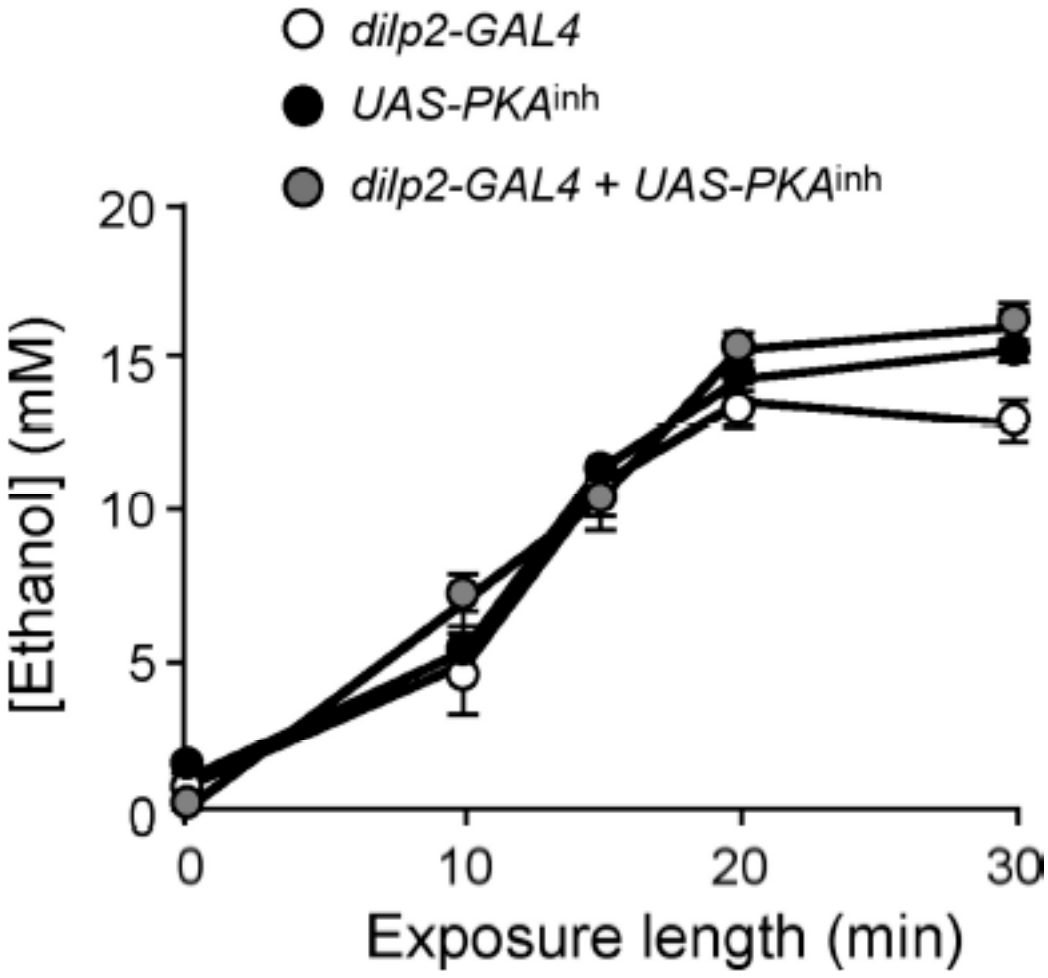

elav-GAL4 3E1 + UAS-InR RNAi n=4
MET (minutes)
25
20
15
10
5
0
1
Genotype
elav-GAL4
elav-GAL4+UAS-InR-RNAi a
UAS-InR-RNAi a
elav-GAL4
elav-GAL4+UAS-InR-RNAi b
UAS-InR-RNAi b
elav-GAL4
elav-GAL4+UAS-InR-RNAi c
UAS-InR-RNAi c

Figure 4


dilp2-GAL4
UAS-PKAinh
dilp2-GAL4 + UAS-PKAinh
[Ethanol] (mM)
20
15
10
5
0
0
10
20
30
Exposure length (min)

Chapter 5: Conclusions and Future Directions

Drosophila melanogaster as a Model Organism for Identifying Genes and Pathways Mediating Ethanol Sensitivity

Beloved by classical geneticists for over a century, *Drosophila melanogaster* has more recently proven itself as a useful model organism in which to study the genes, biochemical pathways, and neuronal circuits underlying various complex behaviors. Much of this usefulness comes from the ease in which *Drosophila* mutants can be generated and studied. Mutants can be generated quickly and easily using EMS mutagenesis or P-element mediated mutagenesis, and the short generation time of *Drosophila* allows the researcher to raise hundreds of mutant flies for testing in a matter of just a few days. Beginning with the pioneering work by Seymour Benzer forty years ago, in which he described a forward genetic behavioral screen through EMS mutagenized fly lines in order to isolate mutant lines with altered behavior in a countercurrent assay (Benzer, 1967), the field of *Drosophila* behavioral study has blossomed, with great advances being made in the study of regulation of circadian rhythm behaviors, learning and memory, courtship behaviors, and, of course, drug-induced behaviors (reviewed in Sokolowki, 2001). Importantly, several of the molecules and pathways implicated in regulating these *Drosophila* behaviors have later been found to have equivalent functional roles in mammals. For example, the various clock genes, first identified in *Drosophila*, including *period*, the first circadian rhythm gene found in any organism (Konopka and Benzer, 1971), have since been shown to underlie circadian rhythms in mammalian systems as well, including humans (Sokolowski, 2001). Similarly,

forward genetic studies implicating *in vivo* roles for the cAMP / PKA signaling pathway in regulating ethanol sensitivity in *Drosophila* (Moore et al., 1998) have since been validated by mammalian studies (Thiele et al., 2000).

While the isolation and identification of individual genes and molecules involved in regulating a complex behavior is informative to a certain degree, what one ideally wishes to do is to organize these various players into a pathway. That is, how do the various molecules identified through forward genetic analyses in *Drosophila* interact with one another in order to regulate the behavior under study? This question can be challenging to answer, but ordering genes into a pathway has certainly been performed with measurable success for certain behavioral processes, especially for circadian rhythm behaviors (Allada et al., 2001), giving hope that studies of ethanol-induced behaviors in *Drosophila* may be similarly fruitful. Indeed, the endeavor of trying to determine what pathway a gene, identified through a forward genetic screen for ethanol-induced behavioral mutants, might be acting through can yield extremely interesting insights. Examples of this include the implication of the cAMP / PKA pathway in *Drosophila* through the study of the *amnesiac*^{cheapdate} mutant (Moore et al., 1998; See introduction) and the implication of the EGFR / ERK pathway in *Drosophila* through the study of *happyhour* mutants (See Chapter 2).

Identifying neuroanatomical loci that are important for regulating drug sensitivity in *Drosophila* has also yielded intriguing results. Studies of ethanol sensitivity in *Drosophila* have pointed to a role for the R2 and R4 neurons of the ellipsoid body (Urizar et al., 2007), while other studies have highlighted a novel role for the PDF-expressing ventral lateral neurons in regulating sensitivity to cocaine-induced behaviors (Tsai et al.,

2004). Conversely, it was found that the mushroom bodies, neuroanatomical structures important in olfactory and courtship conditioning, are dispensable for the regulation of ethanol sensitivity (Rodan et al., 2002). Unfortunately, one of the drawbacks of using fruit flies as a model system is that the functional connectivity of the *Drosophila* central nervous system is not as well mapped out as those of mammalian systems or *C. elegans*, although recent advances in electrophysiological techniques in *Drosophila* promise to improve our understanding of how neural circuits connect and function in the fruit fly (Wilson et al., 2004).

While *Drosophila* is well known for its usefulness in forward genetic screens, recent advances in homologous recombination (Rong and Golic, 2000) and RNA interference (Carthew, 2001; Kalidas and Smith, 2002) technologies have allowed researchers to take a reverse genetic approach to studying the molecular substrates underlying ethanol-induced sedation in *Drosophila*. While forward genetic strategies offer the promise of allowing researchers to isolate novel molecules mediating drug sensitivity with no *a priori* assumptions, reverse genetic strategies allow researchers to test hypotheses regarding specific genes of interest. For example, by taking advantage of double-stranded RNA-mediated gene expression interference (RNAi) strategies which allow for knockdown of endogenous gene expression, Dzitoyeva et al. injected an RNAi directed against the $GABA_BR1$ subunit and found that this reduces the duration of ethanol-induced immobility in an ethanol recovery assay (Dzitoyeva et al., 2003; See introduction). Excitingly, Dietzl et al. have recently reported the generation of a publicly available library of 22,270 transgenic RNAi lines, covering 88% of the predicted protein-coding genes in the *Drosophila* genome (Dietzl et al., 2007), giving researchers a

valuable resource of RNAi lines for use in studying the contributions of specific genes to regulating ethanol sensitivity in *Drosophila*. A word of caution regarding the use of RNAi in *Drosophila* is warranted, however, as two recent studies have shown surprisingly high prevalence of false positives in RNA interference assays due to off-target effects (Ma et al., 2006; Kulkarni et al., 2006).

The use of microarray analysis to identify genes exhibiting changes in expression upon ethanol treatment has also been employed as an alternative method for identifying genes underlying ethanol sensitivity in *Drosophila*. Through such microarray experiments, Urizar et al. isolated *homer* and subsequently found that mutants in *homer* show increased sensitivity to ethanol-induced sedation and impaired development of rapid tolerance to ethanol (Urizar et al., 2007; See introduction). While this study illustrates the usefulness of gene expression profiling in studying the molecules underlying ethanol-induced behaviors, it is important to consider that microarray analyses have a tendency to generate dizzying amounts of data – looking for patterns of gene expression changes among related genes requires a good dose of patience and persistence, but has the potential to be rewarding.

It is worthwhile to emphasize, whether using forward or reverse genetic strategies to study the behavioral effects of ethanol intoxication in *Drosophila*, that behavior can be highly (and frustratingly) variable (A.C. personal experience). Various steps should be taken by the researcher to minimize variability in behavior. As in mice, genetic background can play a large role in affecting observed behavioral phenotypes in flies. As detailed in Chapter 2, several of the mutants isolated in our locomotor tracking screen using a sedation-inducing concentration of ethanol showed strong behavioral phenotypes

when tested in the *w* Berlin genetic background, but not when tested in the *w* Canton-S genetic background. Thus, it is paramount that mutant and control lines be first outcrossed into similar genetic backgrounds before they are tested and compared. In addition, other factors, such as the age and sex of the flies as well as the nutritional and temperature conditions in which they are reared, should be stringently controlled across genotypes in order to minimize variability. Finally, when performing non-automated assays such as the ethanol sedation assay in which the researcher observing the flies may be called upon to make somewhat subjective measurements, it is important that the researcher be blinded to the genotypes of the various flies he/she is observing.

P[GAL4] Collection Screen Mutants: Present and Future

In a screen through ~1500 P[GAL4] mutants to identify genes and molecules involved in mediating the sedative effects of ethanol on behavior in *Drosophila melanogaster*, we (A. Rodan and A.C.) isolated 21 mutants which displayed increased locomotor hyperactivity and/or resistance to ethanol-induced sedation, i.e., 1.4% of the total lines screened displayed a reproducible mutant phenotype in the booz-o-mat locomotor tracking assay using a high concentration of ethanol. After testing these lines post-backcrossing to the parental *w* Berlin strain, I found that approximately half of these lines (11 of the 21 mutant lines) had "lost" their mutant locomotor tracking phenotypes, highlighting the importance of outcrossing mutant lines in order to eliminate unlinked modifiers. Secondary assays conducted on the remaining 10 mutant lines caused me to eliminate two more lines, 4-12a and 6-6, due to apparent alterations in ethanol pharmacokinetics in these two lines compared to controls. Inverse PCR analysis of the

remaining 8 mutant lines led me to set aside two more lines: the P-element insertion in mutant 8-29 is located nowhere near any known genes, while the P-element insertion in mutant 10-187 is upstream of a gene of completely unknown function, gene CG33691. While the identification and putative functions of the genes perturbed by the P-element insertions in 8-29 and 10-187 may be elucidated in the future (the online database of *Drosophila* genes and genomes, Flybase, is continually being updated), I decided that concentrating on a gene whose function was better characterized would be more prudent. Two other P-element insertion lines pulled out of our screen, mutants 7-65 and 10-184, were found to contain P-element insertions in the *white rabbit* / *RhoGAP18B* gene region: these mutants and the *white rabbit* gene were further characterized by a post-doctoral fellow in our laboratory, A. Rothenfluh (Rothenfluh et al., 2006).

Thus, from ~1500 screened P[GAL4] lines, I had narrowed down the number of mutant lines to focus on down to four. After much debate, for reasons described later on in this discussion below, I chose to focus my attention on mutant 17-51, which bears a P-element insertion in the *happyhour* / *CG7097* gene region; the results of these studies are reported in Chapter 3 of this dissertation. The remaining three mutant lines, 17-3, 9-34, and 8-222, are certainly deserving of further characterization, and I shall devote some discussion of these three lines here.

Mutant 17-3 showed a robust increase in hyperactivity over controls at multiple time points during exposure and when tested at a variety of ethanol concentrations. The P-element inserted in mutant 17-3 is located in the CG11033 gene region, which is predicted by Flybase to encode a putative zinc finger-containing transcription factor. It would be interesting to try to ascertain, through gene microarray analysis, gene

expression changes in 17-3 compared to controls in the presence or absence of ethanol exposure. Much can be learned from transcription factor mutants even in the absence of microarray data, as evinced by the results of studies of *Drosophila* mutants affecting expression of another putative transcription factor, *hangover*, and a putative regulator of transcription factors, *dLmo*, both of which have yielded exciting insights into the pathways and neuroanatomical loci underlying drug sensitivity (Scholz et al., 2005; Tsai et al., 2004; See introduction).

A second mutant to come out of the P[GAL4] screen that deserves further characterization is mutant 9-34, which showed increased hyperactivity early on in the locomotor tracking profile but showed decreased locomotor behavior at later time points. The P-element in mutant 9-34 is inserted in between two genes. One gene, *l(2)01289*, encodes a protein predicted to have protein disulfide isomerase activity, while the other gene, *phtf*, encodes a putative homeodomain transcriptional factor. Interestingly, *phtf*, which shows conservation from *Drosophila* to mammals (Manuel et al., 1999), was identified in a misexpression screen as a modulator of axon pathfinding in the *Drosophila* midline (McKinnon et al., 2001), suggesting a role for *phtf* in neurodevelopment.

Finally, mutant 8-222, which showed increased ethanol-induced hyperactivity at various points in the locomotor tracking profile but behaved normally when tested in the ethanol sedation assay, is certainly a promising candidate for future study. With a P[GAL4] CNS expression pattern that is relatively restricted to brain regions including the mushroom bodies, the pars intercerebralis, the median bundle, fan shaped body, and suboesophageal ganglion, 8-222 is a mutant that bears a P-element insertion approximately 100 base pairs upstream of the *Glutamine Synthetase-1* (*GS-1*) gene. GS-1

is one of the two glutamine synthetase isozymes that are responsible for the ATP-dependent amination of glutamate to produce glutamine (Caggese et al., 1994). Studies of hypomorphic *GS-1 Drosophila* mutants showed that GS-1 activity is necessary for the early stages of embryonic development, demonstrating a role for GS-1 in regulating viability (Caggese et al., 1992; Frenz and Glover, 1996). In addition to being involved in nitrogen metabolism and providing glutamine for protein synthesis, glutamine synthetases have also been proposed to modulate neurotransmitter levels through the metabolism of the neurotransmitter glutamate (Caggese et al., 1994). Elucidating how perturbation of glutamine synthetase levels in *Drosophila* might alter behavioral sensitivity to ethanol in otherwise apparently normal flies (data not shown) would likely make for an interesting topic of research.

happyhour **and the EGFR / ERK Pathway**

When, as a significantly younger graduate student, I first embarked on a forward genetic screen to identify *Drosophila* mutants that showed altered sensitivity to ethanol-induced behaviors, I was extremely concerned about how I was to decide which mutant I was to characterize in detail. How was I to choose among the twenty-one mutant lines that came out of this behavioral screen? What criteria should I use? A wise post-doctoral scholar and baymate (D.G.) allayed my concerns with his sage words of advice. He advised that above all, one must choose a mutant with a strong, reproducible phenotype. Secondly, after performing routine secondary assays to assure oneself that trivial explanations, such as altered ethanol pharmacokinetics, are not underlying the observed

robust phenotype, one must identify the gene likely to be involved in mediating the ethanol-induced phenotype and be "excited" by it.

Indeed, *happyhour* (*hppy*) fits the criteria both for "robust phenotype" and for "exciting genotype." Loss-of-function *happyhour* mutants show a strong and readily reproducible resistance to ethanol-induced sedation as measured either in the automated locomotor tracking assay or in the sedation assay. Conversely, overexpressing *happyhour* in neurons results in a robust increase in sensitivity to ethanol-induced sedation. Secondly, inverse PCR analysis revealed that the P-elements inserted in *happyhour* mutants are located in the *CG7097* gene region, which is predicted to encode a MAP4 kinase in the Ste20 group of kinases, an exciting gene in this graduate student's eyes. Rescue experiments went on to show that the *CG7097* / *hppy* gene is indeed responsible for the sedation phenotypes observed in *happyhour* mutants.

In trying to implicate a signaling pathway that *hppy* might act through to alter ethanol sensitivity, the JNK pathway was an obvious candidate. Numerous studies in other systems had placed other Ste20 kinases, including those belonging to the same GCK-1 subfamily of kinases that *hppy* belongs to, firmly as MAP4 kinases in the JNK signaling cascade (Hu et al., 1996; Diener et al., 1997). To my surprise, numerous perturbations of the JNK signaling cascade failed to alter ethanol sensitivity in *Drosophila*, nor did perturbations of another major MAP kinase, p38. Interestingly, I found that manipulations of the third major MAPK cascade, the ERK cascade (specifically the EGFR / ERK cascade), resulted in dramatic changes in ethanol sensitivity. Activation of the EGFR / ERK pathway resulted in decreased sensitivity to ethanol-induced sedation, whereas inhibition of the pathway resulted in the opposite

phenotype. Epistasis experiments using viability and eye development as readouts showed that *hppy* can indeed interact with the EGFR / ERK pathway in mediating these life processes, and support a model in which *hppy* acts as an inhibitor to the EGFR / ERK pathway acting downstream of EGFR but upstream of ERK itself.

One of the most difficult challenges in a graduate career is knowing when to stop, i.e. knowing when one should discontinue performing experiments on a particular project, write up a paper and dissertation, and graduate. The challenge is even more difficult when the project is exciting – with the results and implications of each completed experiment one can imagine two more questions that follow that one wishes to answer. The "*happyhour* - EGFR / ERK" project is no exception. There are a constellation of future experiments and directions that come to mind when thinking about this project. Most of the future directions, which I will detail below, can be performed quite readily with tools available at present. Other questions are more speculative and may require more creative solutions or advances in technology in order to be definitively answered.

One set of questions that are left partially unanswered by my work are the questions of "when" and "where," that is, "when" during development and/or adulthood are *hppy* and the EGFR / ERK pathway functioning to regulate ethanol-induced behaviors and "where" in the nervous system are they functioning to elicit their effects? These questions can be readily addressed by taking advantage of relatively recent advances in the GAL4 / UAS system. Expression of a UAS-transgene of interest can be temporally regulated through the use of the Gal80ts TARGET system, in which transgene expression is induced at higher temperatures (29°C) and repressed at lower temperatures (18°C)

(McGuire et al., 2004), or using the P[Switch] system, in which transgene expression is shut off except in the presence of the drug RU486, which is fed to the flies through their food (Osterwalder et al., 2001). Answering the "when" question would help to parcel out whether the behavioral effects observed in *hppy* mutants and EGFR / ERK pathway transgenic lines are due to active, acute effects of these molecules during adulthood or whether their actions take place during development, perhaps priming the nervous system to be more or less sensitive to the effects of ethanol.

Experiments involving expression of *hppy* or perturbation of EGFR / ERK signaling specifically in the nervous system using the neuronal *elav-GAL4* driver line have gone towards answering the "where" question. Nonetheless, it would be interesting to narrow down the spatial requirements of *hppy* and the EGFR / ERK pathway even further by trying to determine in what subsets of neurons they may be functioning in. Although the functional significance of the various subsets of the *Drosophila* central nervous system are unfortunately not as well understood as those in mammals or *C. elegans*, several studies have provided great insight into which neurons and neuronal circuits are involved in mediating ethanol sensitivity (Rodan et al., 2002; Urizar et al., 2007; Wolf and Heberlein, unpublished observations) and cocaine sensitivity (Tsai et al., 2004). A large number of P[GAL4] lines are available for testing, many of which drive transgene expression in rather specifically defined neuronal subsets. Using these lines to drive expression of *hppy* or EGFR / ERK pathway components, one could hopefully gain a better understanding of where their locations of action lie.

The experiments I've presented in Chapter 3 show that *hppy* can interact with the EGFR / ERK pathway in regulating eye development and viability. I have not shown,

however, that *hppy* can interact with the EGFR / ERK pathway behaviorally in regulating ethanol-induced sedation. While ethanol-induced behavioral experiments demonstrating interaction between a gene of interest and a signaling pathway may be challenging, they certainly can be and have been done successfully (Rothenfluh et al., 2006). Genetic interaction can be determined through behavioral epistasis experiments using null alleles of the genes of interest or by observing whether two minor perturbations in the genes / pathways of interest, which don't by themselves have behavioral phenotypes, show a synergistic behavioral phenotype when they are combined together in the same fly. Unfortunately, epistasis experiments have been hampered by the lack of a null *hppy* allele. This, however, could be remedied by imprecise excision of either of the P-element mutations I have for *hppy*. In addition, while I've shown that *hppy* is sufficient to alter EGFR / ERK signaling, I've not addressed the question of necessity, i.e., is *hppy* necessary for normal EGFR / ERK signaling? This question could be addressed using the fly eye as a readout, where one could examine the effects of reducing *hppy* expression levels, either by using *hppy* loss-of-function mutants or *hppy* RNAi, on fly eye development, specifically in flies that have rough eyes due to decreased EGFR / ERK pathway activity. For example, one would hypothesize that if *hppy* is necessary for normal EGFR / ERK signaling, reducing *hppy* levels in the fly eye might suppress the rough eye phenotypes of flies expressing a dominant negative form of the EGFR (UAS-*egfr*^{DN}).

The genetic interaction between *hppy* and the EGFR / ERK pathway and the involvement of the EGFR / ERK pathway in mediating ethanol sensitivity bring up some intriguing questions. A connection between ethanol its regulation of the EGFR / ERK

pathway had previously been shown through a number of *in vitro* and *in vivo* studies, which found that acute ethanol exposure results in a dramatic inhibition of phosphorylation of both the EGFR and ERK (Chandler and Sutton, 2005; Kalluri and Ticku, 2002; See Introduction). Microarray analyses of brain gene expression of mouse lines showing high or low degrees of ethanol preference showed that the gene expression of a number of genes in the EGFR / ERK pathway, including those coding for EGFR, Grb2, Hras1, Map2K2, and Mapk1/Erk2, are significantly upregulated (for EGFR) or downregulated (Grb2, Hras1, Map2K2, and Mapk1/Erk2) in ethanol preferring mice (Mulligan et al., 2006). In addition, overexpression of an EGFR-specific ligand, transforming growth factor-α (TGF-α), was found to increase ethanol consumption in TGF-α transgenic mice as measured in a two-bottle choice assay (Hilakivi-Clarke and Goldberg, 1995). In my research I have found that activation of the EGFR / ERK pathway results in decreased resistance to ethanol-induced sedation in *Drosophila*, while decreased function of the EGFR / ERK pathway results in increased sensitivity to ethanol-induced sedation. It would be interesting to determine if acute ethanol exposure in *Drosophila* causes decreased phosphorylation levels of EGFR and ERK: antibodies against phospho-ERK and phospho-EGFR are commercially available and can be used for immunoblotting and immunohistochemistry (Ma et al., 2005). As we have presented evidence that *hppy* can inhibit EGFR / ERK signaling, it would be interesting to determine whether ERK phosphorylation levels are decreased in *hppy* mutants in the presence or absence of ethanol treatment. As our data suggests that *hppy* functions downstream of the EGFR, I would hypothesize that EGFR phosphorylation levels should not be altered in *hppy* mutants. The question of where exactly in the EGFR / ERK

pathway does *hppy* function is yet unresolved. As a Ste20 kinase bearing homology to MAP4 kinases, one would hypothesize that *hppy* would act directly upstream of the MAP3 kinase dRaf. This hypothesis, however, has not been specifically tested yet.

Other unresolved questions include how the EGFR / ERK pathway might detect ethanol signals and how it might transduce these signals into behavioral outputs. Both the NMDA receptor and the $GABA_A$ receptor have been implicated in mediating ethanol-induced inhibition of ERK *in vitro*; they are certainly intriguing candidates for ethanol targets upstream of the ERK pathway *in vivo* (Kalluri and Ticku, 2002; Kalluri and Ticku, 2003). There are a multitude of target downstream of the ERK cascade that could possibly transduce the ethanol signal, including various transcription factors, voltage gated ion channels, and second messenger systems (Atkins et al., 1998; Chen et al., 2001). Research to determine the ethanol "inputs" and "outputs" into and out of the EGFR / ERK pathway could very well be a laborious, but worthwhile, undertaking.

It is also worthwhile to consider that while much focus has been on a genetic interaction between *hppy* and the EGFR / ERK pathway, a possible interaction between *hppy* and the JNK pathway has not been completely excluded. It is possible, for example, that *hppy* may interact with the JNK pathway in regulating responses to stress stimuli. Several lines of evidence argue against this possibility, however. For one, *hppy* flies were tested in a variety of stress assays known to activate the JNK pathway, including those measuring the effects of oxidative stress, heat stress, and starvation, and were not found to respond differently from controls in any of these assays (data not shown). Also, *in vitro* work in Hela cells has also demonstrated a lack of involvement of *hppy* in mediating JNK activation in response to stress stimuli such as osmotic stress and the

protein synthesis inhibitor anisomycin (Findlay et al., 2007). Still, as several other GCK-1 subfamily Ste20 kinases have been shown to activate the JNK pathway (Pombo et al., 1995**;** Diener et al., 1997; Hu et al., 1996), a possible *in vivo* role for *hppy* in modulating JNK signaling does probably merit more rigorous testing.

Other unanswered questions spring to mind. What, if any, functional differences exist between the HPPY-PA and HPPY-PB peptides encoded by the two splice variants of *hppy*, *hppy*^{RA} and *hppy*^{RB}? I've shown that expression of the shorter of the two transcripts, *hppy*^{RB}, is sufficient to fully rescue the ethanol resistant phenotypes of *hppy* flies. Perhaps *hppy*^{RA} expression is not important for mediating ethanol-induced sedation in *Drosophila*, or perhaps the two HPPY isoforms are functionally redundant. It would be interesting to test whether expression of a wild-type UAS- *hppy*^{RA} transgene would also be sufficient to rescue the *hppy* mutant phenotype, or alternatively examine whether expressing an RNAi construct targeting the UAS- *hppy*^{RA} transgene could phenocopy the sedation resistant phenotypes observed in *hppy* flies.

Finally, one wonders about how drug specific the phenotype observed in *hppy* flies is to ethanol. Other *Drosophila* mutants with altered ethanol sensitivities have been shown to display altered phenotypes when tested for sensitivity to other drugs such as cocaine and nicotine (Bainton et al., 2005; Rothenfluh et al., 2006) - what about *happyhour* mutant flies? *Hppy*$^{17\text{-}51}$ flies were tested in cocaine sensitivity and nicotine sensitivity assays and were not found to behave differently from controls (data not shown), suggesting that the drug resistant phenotypes observed in *hppy* flies may be ethanol specific. Flies bearing perturbations in EGFR / ERK signaling have not yet been tested for sensitivity to drugs other than ethanol – it would be interesting to determine

whether they too show ethanol-specific phenotypes or whether the phenotypes they display can be generalized to other drugs of abuse.

In conclusion, much like how studies of mutants in the *amnesiac* gene led to the implication of the cAMP / PKA signaling pathway in mediating the *in vivo* behavioral effects of ethanol in flies (Moore et al., 1998; see Introduction), characterizations of sedation resistant mutants in *happyhour* resulted in the implication of another well known signaling cascade, the EGFR / ERK pathway, in mediating ethanol sensitivity in *Drosophila*. It remains to be seen whether the results observed in *Drosophila* will hold true when tested in mammalian systems, but this graduate student is hopeful.

The Insulin/insulin-like Growth Factor Signaling Pathway and Ethanol Sensitivity

In an effort to identify neuroanatomical loci that regulate ethanol sensitivity in *Drosophila*, we expressed an inhibitor of cAMP-dependent protein kinase (*UAS-PKA*^{inh}) in insulin-producing cells (IPCs) using the *dilp2-GAL4* driver line and found that inhibiting PKA activity in the IPCs dramatically increased ethanol sensitivity as measured using the inebriometer. Flies bearing loss of function mutations in the insulin receptor, InR, or the insulin receptor substrate, CHICO, also showed increased sensitivity to ethanol intoxication. Various neuronal perturbations of insulin signaling, through *elav-GAL4*^{3E1} driven expression of an inhibitor of phosphatidylinositol 3-kinase (PI3K), a constitutively active form of the forkhead-related (FKHR) transcription factor hFOXO3a, or an RNA interference transgene targeted against the insulin receptor, all resulted in increased ethanol sensitivity in the inebriometer, showing that inhibition of the InR pathway specifically in the nervous system causes increased sensitivity to the intoxicating

effects of ethanol. One caveat to keep in mind is that the *UAS-InR*^{RNAi} construct, which was constructed and injected into flies by members of the Heberlein lab, has not yet been characterized in terms of its efficacy in knocking down InR expression levels. This should be done before this fly line is used for further experiments.

This data connecting the insulin/insulin-like growth factor signaling (IIS) pathway to the regulation of ethanol sensitivity leaves us with several intriguing unanswered questions. As with the EGFR / ERK pathway (described above), it would be interesting to use recent advances in GAL4/UAS system technology to gain a better understanding of the spatial and temporal requirements of the IIS pathway in regulating ethanol sensitivity. Temporal requirements could be addressed through utilization of the RU486-mediated P[Switch] method of transgene induction or through the temperature dependent TARGET system (Osterwalder et al., 2001, McGuire et al., 2004) in order to drive various IIS pathway transgenes, including *UAS-P60*, *UAS-hFOXO*^{TM}, and *UAS-InR*^{RNAi}. The insulin receptor is broadly expressed in the *Drosophila* nervous system (Garofalo and Rosen, 1988); perhaps through the use of more specific neuronal drivers we could get a better understanding of what neuroanatomical targets of insulin signaling are relevant for regulating ethanol sensitivity in flies.

While the neurosecretory insulin producing cells (IPCs) are clearly involved in regulating ethanol sensitivity, it has not yet been determined through which secreted molecule(s) they are mediating their effects. The most obvious candidate, of course, is insulin, which has been shown to be secreted from the IPCs (Rulifson et al., 2002), and whose secretion has been shown to be positively regulated by the cAMP / PKA signaling pathway (Lester et al., 1997). Thus, a reasonable hypothesis is that inhibiting PKA

activity in the IPCs through *dilp2-GAL4* expression of *UAS-PKA*^{inh} results in decreased insulin secretion from the IPCs, which consequently leads to increased ethanol sensitivity in the inebriometer. Do *dilp2-GAL4 + UAS-PKA*^{inh} flies show decreased levels of secreted insulin? As the IIS pathway has been shown through numerous studies to be a positive regulator of body size in *Drosophila*, the observation that *dilp2-GAL4 + UAS-PKA*^{inh} flies are 10-15% smaller than control flies when measured for total body protein content (data not shown) is certainly supportive of this hypothesis. It would be helpful to make more quantitative measurements of levels of circulating insulin in *dilp2-GAL4 + UAS-PKA*^{inh} flies. Insulin levels can be quantitatively measured through radioimmunoassay or ELISA using anti-Dilp2 antibodies (Rulifson et al., 2002). Alternatively, one can indirectly measure insulin levels by quantitatively measuring levels of the hemolymph sugars glucose and trehalose, as inhibition of insulin secretion has previously been shown to increase the circulating levels of these sugars (Rulifson et al., 2002).

Like *dilp2-GAL4 + UAS-PKA*^{inh} flies, loss-of-function mutant flies in the *amnesiac* (*amn*) gene, which encodes a peptide with homology to mammalian PACAP, also show increased sensitivity to ethanol intoxication as measured in the inebriometer (Moore et al., 1998; See introduction). In mammals, PACAP, acting through the PACAP receptor PAC1 and the cAMP / PKA signaling cascade, has been shown to potentiate glucose-stimulated insulin secretion (Jamen et al., 2000; Jamen et al., 2002). An intriguing hypothesis is that the *amnesiac* gene product may potentiate insulin release in *Drosophila* as well, and that that the increased ethanol sensitivity observed in *amn* mutants is due to impaired insulin secretion. It would be interesting to determine whether *amn* mutant flies have decreased levels of circulating insulin, and whether the ethanol

sensitive phenotypes of *amn* mutants can be rescued through transgenic expression of insulin, e.g. through heat shock inducible expression of insulin using a *hs-dilp2* construct (Rulifson et al., 2002).

Of course, one would like to determine whether the IIS pathway regulates ethanol sensitivity not only in *Drosophila*, but in mammalian systems as well. Interestingly, a study in transgenic mice suggests this may the case. In this study, it was found that transgenic mice overexpressing insulin-like growth factor I (IGF-I), which positively signals through the IIS pathway, displayed decreased ethanol sensitivity to the sedative/hypnotic effects of ethanol as measured in a loss-of-righting reflex assay and also showed a failure to acquire chronic ethanol tolerance after repeated ethanol administrations (Pucilowki et al., 1996). Conversely, transgenic mice overexpressing IGF binding protein 1, which inhibits the *in vivo* actions of IGF-I (D'Ercole et al., 1994), showed the opposite phenotypes: increased sleep time in the loss-of-righting reflex assay and increased chronic ethanol tolerance (Pucilowski et al., 1996). It would be interesting to determine whether future studies in transgenic and knockout mice bearing perturbations in the IIS pathway will yield similarly promising results.

Summary

One cannot help but be impressed by how far the field of ethanol research in *Drosophila melanogaster* has progressed over the span of just a few years. Practically unknown as a model system for ethanol study just a decade ago, the fruit fly has since proven itself as an invaluable tool for the identification and characterization not just of isolated genes and molecules involved in mediating ethanol's constellation of behavioral

effects, but as a tool for illuminating the neuroanatomical loci and biochemical pathways that underlie ethanol sensitivity as well. The experimental results described in this dissertation further our understanding of the various molecules, including Happyhour, and signaling cascades, including the IIS and EGFR / ERK pathways, that play a part in regulating ethanol-induced behaviors in *Drosophila*. Recent advances in biotechnology promise to increase our understanding of drug induced behaviors in *Drosophila* not just through traditional forward genetic screens but through reverse genetic and microarray approaches as well. These and future results garnered in *Drosophila*, complemented by those elucidated from experiments in *C. elegans* and mammalian systems, slowly but surely will provide us with the puzzle pieces to help us understand the molecular mechanisms underlying ethanol-induced behaviors and ultimately, alcohol addiction.

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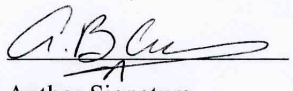

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