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Molecular and genetic mechanisms of ethanol tolerance in the fruit fly

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Dedication

To Sudha amma and Ravi appa

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Molecular and genetic mechanisms of ethanol tolerance in the fruit fly

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Tolerance to the sedative effects of ethanol is an acute physiological change that can lead to more chronic phenotypes such as dependence and addiction. Ethanol tolerance in the adult nervous system of the fruit fly, *Drosophila melanogaster*, is dependent on the calcium-activated potassium channel (BK) gene, *slowpoke*. This gene is highly conserved between flies and humans and critically involved in neuronal communication in the brain, where it modulates action potential duration and regulates the firing rate of neurons. Adult Drosophila acquire rapid functional tolerance to ethanol after a single sedation due in part to a transcriptional up-regulation of the *slowpoke* message.

Genes can be regulated at multiple levels, for instance, at the level of transcription, level of mRNA splicing, mRNA editing, post-translational modifications to the protein and protein localization. Using a heat inducible transgene expressing *slowpoke* in a null mutant background, I show that flies are able to acquire tolerance in the absence of

transgenic induction. This suggests that *slowpoke* mediated rapid tolerance to ethanol involves a transcription-independent mechanism.

Regulation of gene expression involves post-translational modifications to histones which cause chromatin decondensation and recruit transcription factors at the promoter to initiate transcription. Using the chromatin immunoprecipitation assay followed by real-time quantitative PCR, I show that the *slowpoke* promoter has a distinct spatio-temporal pattern of histone acetylation following a single sedative dose of ethanol. Increased acetylation at conserved control elements in the promoter is responsible for the transcriptional up-regulation of the gene following ethanol sedation.

It is well known that anesthetics and abused drugs preferentially modulate specific molecular pathways in defined regions of the brain. In this study I have identified brain structures where *slowpoke* induction may play a role to elicit tolerance. Using the binary GAL4/UAS system to express *slowpoke* in different parts of the Drosophila nervous system I show that *slowpoke* induction in the mushroom bodies of the adult fly brain, a region critical for olfactory learning and memory, is important for ethanol tolerance. A survey of brain structures mediating drug tolerance in the genetically tractable Drosophila could potentially reveal evolutionarily conserved molecular pathways regulating tolerance.

Using temperature sensitive mutants to block neuronal transmission I show that tolerance is a cell autonomous property of the nervous system that is independent of synaptic communication and not an emergent property of the whole brain. I also show that to elicit tolerance the presence of drug is necessary and reduced neuronal signaling does not phenocopy or mimic tolerance.

I have identified Drosophila *shibire* as a critical gene mediating rapid tolerance to ethanol in flies. This gene is the Drosophila homolog of *dynamin* and regulates vesicle recycling at the synapse and neuronal communication in the brain. Two independent alleles of *shibire*, shi^{ts1} and shi^{ts2} are incapable of acquiring tolerance to ethanol.

Increased activity of SLOWPOKE channels is predicted to enhance the firing frequency of neurons. This may augment the recovery of flies from sedation leading to the observed behavioral tolerance. DYNAMIN also functions at the synapse to mediate rapid neurotransmission and critically modulate neuronal excitability. This suggests that rapid tolerance to ethanol sedation may be a neuroadaptive state regulated by homeostatic changes in neuronal excitability that lead to faster recovery from sedation.

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Chapter 1: General Introduction

Ion channels are critical modulators of neuronal activity

Ion channels underlie the character of tissues composed of nerve cells, muscle cells and endocrine cells and regulate their function thereby impacting behavior. Higher organisms are endowed with a complex nervous system whose functions range from a simple monosynaptic spinal reflex to higher brain functions such as learning and memory. Neural signaling is finely controlled by ion channels that are gated by extra-cellular ligands, voltage and intracellular messengers. The cohort of ion channels a neuron expresses influence the electrical properties of the neuron and effect the desired outcome based on its function, be it regulated neurotransmitter release at the synapse, information processing in the dendrite or coincident signal processing in the auditory system.

We are interested in studying the acute effects of volatile anesthetics and abused drugs on the nervous system of *Drosophila melanogaster*. Drugs and anesthetics cause perturbations in neuronal activity. The nervous system responds by regulating its excitability so that homeostasis is maintained. Such neural plasticity is dependent on the activities of ion channels that can dynamically change the excitability of the system. Long-term or chronic abuse of drugs leads to permanent adaptation by the nervous system leading to addiction (Koob et al., 1998). We have demonstrated that the transcriptional regulation of the *slowpoke*, Ca^{2+}-activated K^+ channel gene is a critical modulator of neuronal excitability and is necessary for the acquisition of tolerance to volatile anesthetics and abused drugs (Ghezzi et al., 2004; Cowmeadow et al., 2005; Cowmeadow et al., 2006; Wang et al., 2007). Let us briefly look at the importance of ion

channel regulation especially the BK channel and the role it plays in regulating neuronal excitability in Drosophila.

The voltage gated potassium channels

Ion channels are named based on the ions they conduct such as sodium, potassium, calcium and chloride. They are also classified based on their activation and gating properties such as ligand gated, voltage gated, cyclic nucleotide gated and calcium activated. Ion channels are highly specialized plasma membrane proteins that act as molecular conduits for ions to flow down their electrochemical gradient selectively and rapidly. The voltage-gated potassium channels are a very diverse family of related proteins which have evolved to have the greatest diversity among ion channel genes. Mammals have nine Na^+ channel genes, ten Ca^{2+} channel genes, but over seventy-five genes encoding K^+ channels. In Drosophila only two Na^+ channel genes have been identified while twenty-seven K^+ channel genes have been found. Seventy-six K^+ channel genes are known in *C. elegans*, which surprisingly has no Na^+ channel genes (Hille, 2000).

Potassium channels are primarily involved in the repolarization of membrane action potentials and mediate various physiological roles in cells. The great diversity among the K^+ channels enables a cell to appropriately select the correct set of channels to adapt to its specific needs. Alternative splicing and the ability to hetero-multimerize lends more complexity accounting for phenotypic variation that influences behavior. Potassium channels are known to modulate neural activity in diverse surroundings and in complex ways. To fire an action potential is an "all or none" trait, but by expressing certain potassium channels in distinct amounts at specific time-points, the cell dictates the shape and frequency of the action potentials. These are crucial decisions in an axon, a synapse,

the hippocampus or cardiac muscle. Thus, the ability for the cell to modulate its excitation, fine tune its circuitry and polish its response would have placed a greater evolutionary stress, accounting for such a great variety in these channel proteins. Since all neurons express K^+ channels, it is not only their presence that distinguishes a cell, but rather the choice of the channel and its spatio-temporal expression.

Potassium channels in Drosophila

Molecular genetic methods and expression cloning have led to the identification of many K^+ channel genes in Drosophila. They include the *Shaker* family of K^+ channels involved in action potential repolarization with Drosophila *Shaker* (Kv) being the first cloned potassium channel gene from any species. There are three other evolutionarily conserved members *Shab, Shal* and *Shaw*. These channels mediate transient (Ia) and delayed rectifier K^+ currents (Ik) (Papazian et al., 1987). Another delayed rectifier group of K^+ channels, the *eag* family of genes now includes three evolutionarily conserved subfamilies *eag, erg* and *elk* (Warmke and Ganetzky, 1994; Titus et al., 1997; Wang et al., 1997). Members of this subfamily share characteristics with both the Kv *Shaker* type channels and cyclic nucleotide gated cation channels (CNG) and have a C-terminus cyclic nucleotide binding domain (Warmke and Ganetzky, 1994). Single genes in Drosophila represent all three subfamilies of *eag*. Changes in voltage and intracellular calcium concentrations activate a novel family of potassium channels, the Ca^{2+}-activated K^+ channels. This family is further classified based on channel conductance and includes BK or Maxi K (100-300 pS), IK (25-100 pS) and SK (2-25 pS) channels (Marty, 1981; Gardos, 1958; Logsdon et al., 1997; Kohler et al., 1996; Ishii et al., 1997; Joiner et al., 1997; Sah, 1996). Drosophila has a single gene encoding for BK (*slowpoke*) and SK channels.

BK channels and their functional diversity

The Ca^{2+}-activated K^+ channel α-subunit of the BK sub-family was first cloned and expressed from Drosophila. It is encoded by the *slowpoke* locus (Atkinson et al., 1991; Adelman et al., 1992). Four α subunits come together to make a functional channel with each subunit having seven transmembrane domains (Meera et al., 1997). These channels have the unique characteristic of being activated allosterically by membrane depolarization alone or intracellular calcium alone but the open probability (P_O) is very negligible unless they both activate the channel synergistically (Magleby, 2003). BK channels perform many physiological functions including repolarizing the membrane, modulating neurosecretion, regulating smooth muscle tone, shaping action potential waveforms, orchestrating spike frequency adaptation, contributing to electrical tuning of cochlear hair cells, playing a role in sperm activation, controlling circadian clock behavioral rhythms and output (Robitaille et al., 1993; Yazejian et al., 1997; Brayden and Nelson, 1992; Nelson and Quayle, 1995; Rosenblatt et al., 1997; Navaratnam et al., 1997). The BK channels have immense functional diversity in that different concentrations of calcium can activate BK channels in specialized cell types such as hair cells (Fettiplace and Fuchs, 1999).

The functional diversity of BK channels is also enhanced by accessory β subunits (β 1-4) which affect the voltage and calcium sensitivities of channel activation and gating. β1 has been shown to contribute to the calcium sensitivity of BK channels in inner ear hair cells, mediate N-type inactivation of the α subunit in hippocampal and chromaffin cells and mediate estradiol activation of the BK channel (Fettiplace and Fuchs, 1999; Cox and Aldrich, 2000; Brenner et al., 2000; Orio et al., 2002; Orio and

Latorre, 2005; Bao and Cox, 2005; Wallner et al., 1999). The BK channel genes undergo alternative splicing which generates many different transcripts that differ in their calcium sensitivities and gating properties (Tseng-Crank et al., 1994; Lagrutta et al., 1994; Adelman et al., 1992; Yu et al., 2006). BK channels are also regulated by protein kinases including protein kinase A (PKA), protein kinase G (PKG), protein kinase C (PKC) and Ca^{2+}/calmodulin-activated protein kinase II (CAMKII). Phosphorylation fine-tunes the responses of these channels to intracellular calcium and membrane voltage (Schubert and Nelson, 2001; Tian et al., 2001; Liu et al., 2006).

We are interested in the transcriptional regulation of the Ca^{2+}-activated K^+ channel (BK) gene, *slowpoke*, in response to volatile anesthetics and drugs of abuse in the fruit fly, *Drosophila melanogaster*. Volatile anesthetics and abused drugs such as ethanol act on a number of ion channels and regulate synaptic activity and neuronal homeostasis. The BK channel is modulated by ethanol at both the transcriptional and post-translational level in different biological systems. Let us briefly review what is known about ethanol's effects on ion channels in general and then look at BK channel regulation.

Effects of ethanol on ion channel function

Ethanol pleiotropically affects different ligand and voltage gated ion channels. The $GABA_A$ and glycine receptor sub-types are activated by ethanol. With $GABA_A$ different ethanol phenotypes can be mediated by different subunit combinations, for example "loss-of-righting reflex" (LORR) is associated with the α1 subunit and ethanol induced locomotion by the α5 subunit. Similarly ethanol has also been shown to activate glycine receptors by increasing channel open probability (Beckstead et al., 2000; Crabbe et al., 2006; Boehm et al., 2004).

Alcohol also seems to potentiate the 5HT-3 ionotropic cation receptor. Ethanol increases the agonist, 5-hydroxy tryptamine, potency and keeps the channel stabilized in the open state (Lovinger and White, 1991; Narahashi et al., 2001; McBride et al., 2004). With respect to nicotinic acetyl choline receptors, alcohol has a differential effect; potentiating the predominant neuronal α4 β2 receptors, while having very little effect on the α3 variant present in peripheral ganglia (Zuo et al., 2002; Narahashi et al., 2001).

Ethanol's actions on excitatory receptors and ion channels is predominantly inhibitory. Glutamate is the major excitatory neurotransmitter in the CNS and acts via metabotropic as well as three ionotropic receptors classified as NMDA (N-methyl D-aspartic acid), Kainate and AMPA (α-amino-3-hydroxy-5-methylisoxazole-4-propionic acid) receptors. Ethanol has been shown to inhibit NMDA receptors (Lovinger et al., 1989; Wirkner et al., 1999). Deletion of the epsilon subunit of the NMDA receptor prevents acquisition of tolerance for the LORR in mice (Sato et al., 2006). Voltage gated Ca^{2+} channels, L type, are inhibited by ethanol in neurohypophysial terminals thereby affecting hormonal release (Wang et al., 1994).

Post-translational and transcriptional regulation of BK channels by ethanol

Ethanol regulates BK channels at the transcriptional and post-translational level in invertebrate and vertebrate model systems. Using a behavioral assay for tolerance in the invertebrate model, *Drosophila melanogaster*, we observed a transcriptional up-regulation of the *slowpoke*, Ca^{2+}-activated K^+ channel gene following ethanol sedation leading to tolerance. Transgenic induction of a *slowpoke* cDNA in mutant (slo^4) flies phenocopied tolerance (Cowmeadow et al., 2005; Cowmeadow et al., 2006). Tolerance to ethanol sedation in flies is a neuronal phenotype.

Ethanol post-translationally affects BK channels in both vertebrate and invertebrate model systems. In vertebrates ethanol positively regulates BK channel function by increasing open probability (P_O) of the channels. In other cases ethanol is shown to inhibit BK channels. Ethanol potentiates BK channels in neuronal preparations such as dorsal root ganglia (DRG) neurons where it is postulated to potentiate analgesia due to reduced excitability of sensory neurons (Gruss et al., 2001). On clonal pituitary (GH3) cells ethanol increases BK currents and this effect is mediated by PKC (Jakab et al., 1997). BK channels in rat neurohypophysial terminals are activated in the short term while in the long term they exhibit decreased sensitivity exhibiting tolerance to this effect (Knott et al., 2002; Pietrzykowski et al., 2004). The activating effect of ethanol observed in native membranes is also seen in heterologous expression systems. Cloned channels from oocytes and artificial lipid planar bilayers suggesting that this is a direct effect of ethanol on the channel (Dopico et al., 1998; Chu et al., 1998). It has been shown that the membrane phospholipid constitution affects ethanol actions on BK channels. Reconstituting human BK channels in phospholipid bilayers shows that

phosphatidylserine (PS) which is involved in membrane bound signaling potentiates BK channel activity as opposed to phosphatidylcholine (Crowley et al., 2005).

Recently it has also been shown that ethanol actions on human BK channels can be modulated by β subunits in such a way that β1 to a large extent and β4 to a small extent reduce the ethanol potentiation of the channel (Feinberg-Zadek and Treistman, 2007). Given that β subunits also modulate voltage and calcium sensitivities of these channels this can lead to very specific spatial alterations in BK channel activity. BK channels are inhibited by ethanol in aortic smooth muscle leading to arteriolar constriction (Dopico, 2003).

In the invertebrate model *Caenorhabditis elegans*, ethanol interacts with BK channels to regulate behavioral effects. *Caenorhabditis elegans* BK channel mutants show resistance to ethanol sedation. In this organism, BK channels are implicated in mediating the intoxicating effects of ethanol via activation and thus repolarizing the membrane (Davies et al., 2003).

EPIGENETIC MODIFICATIONS DYNAMICALLY REGULATE GENE EXPRESSION

Neuroplasticity is an integral characteristic of the brain that regulates the acquisition of memory and allows us to adapt to changes in the environment. Abused drugs can affect dopaminergic reward circuits in the brain leading to long term stable changes and addictive states. Neural plasticity requires changes in gene expression and drugs of abuse have been shown to modify the promoters of genes by histone modifications and recruitment of transcription factors including the cAMP response element-binding protein (CREB). It is important to characterize the molecular pathways converging on downstream targets at the transcriptional level in order to understand the basis for drug tolerance and addiction. We have shown that the promoter of *slowpoke* is dynamically modified via histone acetylation leading to transcriptional up-regulation of the gene leading to benzyl alcohol tolerance (Wang et al., 2007). In this study I asked whether similar mechanisms are invoked to mediate ethanol tolerance in flies.

What are epigenetic modifications?

The prefix "epi" in Greek stands for "above" or "over" and "genetic" stands for "relating to genes or heredity". Therefore etymologically it refers to an accessory or additional method of biological inheritance which does not involve the DNA. The scientific community uses the word to describe a heritable change in gene expression influencing the phenotype that does not involve a modification of DNA sequence or mutation (Holliday, 1987). Not everyone uses the term to mean a heritable change. Some classify it as "mimicking" heritable change as some of the epigenetic modifications are ephemeral and do not last the lifetime of the animal.

Epigenetic changes fall under an umbrella that includes covalent post-translational

modifications to histones and transcription factors, energy-dependent chromatin remodeling, DNA modification by methylation at cytosine residues and role of small non-coding RNAs to control gene expression. All of these changes influence the structure of chromatin regulating gene expression from the locus or chromosome.

Modifications such as DNA methylation and histone lysine methylation can be stable through mitotic cell divisions. Histone lysine methylation at H3K9 is involved in maintaining heterochromatin through the recruitment of heterochromatin protein 1 (HP-1) and this is maintained in the daughter cells. Similarly, studies done with imprinted loci show that DNA methylation patterns are re-established in the developing embryo even though they are removed in primordial germ cells (Li, 2002; Davis et al., 2000; Allegrucci et al., 2005). Methylation of DNA has a two-fold effect; directly hindering association of activators and indirectly through recruitment of histone deacetylases (HDAC), co-repressors and other heterochromatin associated proteins thereby contributing to the stable maintenance of heterochromatin through generations (Nan et al., 1998; Klose and Bird, 2006). It has been observed that maternal grooming in rats can alter the offspring glucocorticoid receptor promoter in the offspring (Weaver et al., 2004). Thus we see that a modified chromatin state can persist through subsequent generations and can be inherited through the germline.

Most of the histone modifications are dynamic and reversible and so unlikely to be carried over through the germ line. These changes occur in non-germline cells such as neurons leading to both short term and long term changes in gene expression. Because the chemical moieties involved are the same and the consequences of the change are the same, the term epigenetics was used to refer to these changes even though they occurred in non germline cells and are not inherited. This leads to confusion regarding use of the

word "epigenetic". For example DNA methylation affects gene regulation both directly and indirectly. The indirect mechanism is by recruitment of HDACs and heterochromatin associated proteins including HP1 which in-turn modify histones and repress transcription (Fischle et al., 2005). This can be viewed as a propagation of a stable chromatin state through generations even though the changes themselves are dynamic, happening during the lifetime of the animal.

Bird (2007) in his essay refines the definition as follows, “the structural adaptation of chromosomal regions so as to register, signal or perpetuate altered activity states” (Bird, 2007).

Why are epigenetic mechanisms important for normal development? Consider the fact that there is only a 4-5 fold increase in gene number between unicellular yeasts and multicellular mammals. This modest increase has to account for the functional variation and complexity required in about 200 different cell types. Therefore epigenetic regulation of the genome is an important mechanism underlying normal development.

Nucleosomal structure and chromatin remodeling

The fundamental unit of chromatin is the nucleosome. It is made up of core histones H2A, H2B, H3 and H4 forming an octamer around which 146 bp of DNA is wrapped (Luger et al., 1997). The boundary between two nucleosomes is anchored by histone H1. Some of the most well studied epigenetic mechanisms influencing chromatin structure include covalent modifications such as DNA methylation at CpG residues and post-translational histone modifications at their N- and C- termini at more than 60 residues by different classes of enzymes (Klose and Bird, 2006; Kouzarides, 2007). These modifications cause changes in chromatin structure engendering chromatin

remodeling. The DNA is rendered more or less accessible to other transcription factors that affect transcriptional output from the locus.

Chromatin remodeling can also occur by an ATP dependent process involving "remodelers". These classes of proteins remodel nucleosomes in at least four different ways: sliding the histone octamers exposing the DNA, ejecting the whole octamer thus exposing the DNA, destabilizing the nucleosome by removing H2A-H2B dimers leaving H3-H4 dimers behind, and replacing H2A-H2B dimers with variant H2A.Z-H2B dimers which are associated with gene activation (Cairns, 2007). A number of remodeling proteins have been characterized in eukaryotes including the well studied Switch/Sucrose Non-Fermentable (SWI/SNF) class of remodelers. Another family of remodelers is the Imitation SWI (ISWI) family of remodelers. These proteins have unique differentiating characteristics. The SWI/SNF family of proteins have bromodomains which target acetylated histone tails and they are found to be localized at those regions. The ISWI class target DNA and histone tails via two domains, the SANT (Swi3. Ada2, N-Cor and TFIIIB) and SLIDE (SANT-like) domains (Saha et al., 2006). In general all remodeling proteins have ATPase activity enabling them to change nucleosomal structure. The Drosophila nucleosome remodeling factor (NURF) is an ISWI containing ATP-dependent remodeling complex. NURF has been implicated in crucial processes linked to metamorphosis and steroid signaling (Badenhorst et al., 2005).

Chromatin remodeling has been shown to affect processes as diverse as neural cell fate determination, synaptic plasticity, learning and memory, chronic stress, circadian rhythms and cocaine-induced neuronal plasticity (Hsieh and Gage, 2005; Groth et al., 2007; Guan et al., 2002; Levenson et al., 2004; Tsankova et al., 2006; Naruse et al., 2004; Kumar et al., 2005).

Histone modifications and their functions

Various histone post-translational modifications (PTMs) have been characterized that include acetylation, methylation, phosphorylation, ubiquitination, sumoylation, ADP-ribosylation and proline isomerization (Kouzarides, 2007). Acetylation and phosphorylation have been implicated in activation while sumoylation has been implicated in repression. Ubiquitination, methylation and proline isomerization cause either activation or repression in a context specific manner. A key substrate in histone biochemistry is lysine which undergoes acetylation, methylation, ubiquitination and sumoylation. Added complexity is generated when we consider that lysine methylation of histone H3 at specific residues (K4, K36 and K79) causes activation. But methylation just a few residues over at K9 and K27 causes transcriptional repression. Moreover these residues can be mono, di or tri methylated adding another layer of regulation (Lachner et al., 2003). These changes are dynamic with deacetylases, serine-threonine phosphatases, lysine demethylases, deiminases and ubiquitin proteases acting to reverse the corresponding modifications.

The different histone classes H1, H2, H3, H4 are encoded by multiple genes. In addition different histone variants can also be utilized for different chromatin states. In the case of histone H3, H3.1 and H3.2 differ by a single amino acid while H3.2 is enriched in modifications involved with gene silencing H3.1 is enriched in active post-translational modifications (Hake and Allis, 2006).

The most important function of histone modifications is condensation or de-condensation of chromatin thereby regulating access to the DNA by transcription factors. For example acetylation neutralizes the basic charge of lysine directly promoting

unraveling of the DNA by minimizing nucleosomal contacts. In addition transcription factors or activators are recruited to the modified histones and the associated DNA. They can regulate transcription from the locus in a number of different ways. They can initiate ATP-dependent remodeling altering the chromatin structure to facilitate increased transcription via assembly of the RNA polymerase II complex. They can also bring with them enzymatic activities which further modify neighboring histones propagating the unraveling of associated DNA. In many instances transcriptional activators recruit co-activators leading to more complex regulation and control of transcription. The regulation of gene expression depends on the PTMs to the histones and mutually antagonistic PTMs can affect expression from the locus accordingly. For example the stable tri-methylation of lysine K9 of histone H3 recruits a protein HP-1 which is important for maintenance of heterochromatin, but an adjacent dynamic phosphorylation of serine at the next residue, Ser10, inhibits the interaction so that cells can proceed to the M phase of the cell cycle (Fischle et al., 2005). Histone modifications can be context specific, for instance H3K9 methylation is associated with transcriptional activation when it is present at open reading frames (ORF) within the coding region of the gene but during formation of pericentromeric heterochromatin it is repressive in nature (Berger, 2007). The specificity and complexity of such modifications have been postulated to form a code with certain combinations of modifications occurring in a spatio-temporal fashion causing discrete and specific outcomes from the locus (Jenuwein and Allis, 2001; Strahl and Allis, 2000; Turner, 2007).

Let us briefly review an important modification of histone N- termini, acetylation and its impact on gene regulation and drug induced neural plasticity.

Histone acetylation

A well characterized histone post-translational modification is acetylation, causing increased transcription from the locus. This activity is mediated by histone acetyltransferases (HATs) that are enzymes that acetylate specific lysine tails mostly in the N- termini of core histones (H2A, H2B, H3 and H4) causing an increase in transcription. Some of the well studied lysine residues undergoing acetylation include K5, K8, K12, K16 in histone H4 and K9, K14 and K18 in histone H3 (Kouzarides, 2007). The acetylation sites are dotted on the N- terminal tails of histones which are more accessible for modification. Recently acetylation has been seen even in the core domain of histone H3, K56, associated with activation (Han et al., 2007; Xu et al., 2005). The activity of HATs is reversed by a different enzyme group, histone deacetylases (HDACs). HDACs cause a decrease in transcription by removing acetyl groups from histones. HATs are divided into three major families Gcn5-associated N-acetyltransferase (GNAT), MYST family (named after its founding members MOZ, Ybf2/Sas3, Sas2, Tip60) and CREB binding protein/p300 (CBP/p300). The HATs are known to acetylate more than one lysine residue with only a few showing specificity (Sterner and Berger, 2000). HDACs have been grouped into four classes: classes 1, 2 and 4 are similar requiring zinc for activity whereas class 3 HDACs or sirtuins (SirT2) are structurally unique requiring NAD for deacetylase activity (Hildmann et al., 2007; Vaquero et al., 2006). Generally transcriptional activators and co-activators recruit HATs whereas repressors and co-repressors recruit HDACs.

Histone acetylation and neural plasticity

Histone acetylation has been implicated in numerous brain regulatory pathways.

An example is the circadian clock in mammals which requires the precise transcriptional regulation of several transcription factors that cycle accordingly. The acetylation state of the promoter of clock genes such as *period* were also shown to cycle influencing the circadian rhythm (Naruse et al., 2004). In fact one of the key regulators of the circadian cycle, the CLOCK protein has a HAT domain enhanced by BMAL1 binding (Doi et al., 2006). Here we see a transcription factor, CLOCK, regulating acetylation without recruiting a separate co-activator HAT such as CBP/p300.

A well characterized and studied mechanism of cellular plasticity in the brain is regulation of memory formation by long-term potentiation (LTP) and long-term depression (LTD). LTP and LTD are critical in all types of memory formation including drug reward related memories mediated by dopaminergic systems in the brain (Hyman et al., 2006). Studies in Aplysia have shown that acetylation/deacetylation at the immediate early gene C/EBP regulates long term facilitation or depression (Guan et al., 2002). In mice it has been observed that the HAT activity of CBP/p300 and its recruitment by CREB is critical for short-term to long-term memory consolidation. In mice that have a mutant CBP with loss of HAT activity, the phenotypic defect can be restored by administration of the HDAC inhibitor, Trichostatin A (Alarcon et al., 2004; Korzus et al., 2004). It has been observed that changes in gene expression induced by epigenetic modifications can also affect subsequent generations. Maternal grooming in rats affects the glucocorticoid receptor promoter in offspring in response to stress. This effect is due to acetylation changes at the promoter and is potentially reversible (Weaver et al., 2004).

Histone modification and drug addiction

Drug induced changes in gene expression underlie short-term phenotypes that include tolerance and sensitization and in the long-term can cause stable alterations in

synaptic structures in response to chronic drug use, leading to an addictive state (Nestler and Aghajanian, 1997). Understanding the molecular circuitry and neurotransmitter systems involved in drug addiction is essential to combat the problem. Repeated drug intake involves release of dopamine from cells in the ventral tegmental area (VTA) into the prefrontal cortex (PFC), striatal complex and amygdala (Kelley, 2004; Nestler, 2005). Drugs can take over this pathway that normally responds to motivationally relevant biological stimuli such as food and sex. There are important differences between the two stimuli. Motivational stimuli have physiological checkpoints which homeostatically regulate drive whereas drugs can drive the dopamine release beyond normal levels leading to compulsive behaviors including craving, dependence, withdrawal and addiction.

Abused drugs increase dopamine responses in specific areas of the brain. This increase can cause dopamine receptor activation, especially D1 receptors. D1 receptor activation in the striatum and cortex can lead to important functional consequences such as increases in cAMP, cAMP-dependent protein kinase (PKA), cAMP response element binding protein (CREB) and DeltaFosB activity. Transcription factors such as CREB and DeltaFosB are master switches that initiate transcription of many genes including growth factors, enzymes and other transcription factors (Nestler, 2004). Ultimately these changes can cause stable structural synaptic modifications leading to behavioral neuroplasticity and addiction.

Transcription factors recruit co-activators which carry HAT activities and modify the structure of chromatin. These changes can lead to regulation of gene expression and in the case of drug addiction when the stimulus is persistent lead to stable alterations in the neural circuits and neuronal plasticity. Cocaine administration to adolescent rats

resulted in increased responses to cocaine in adulthood accompanied by a decrease in histone H3 methylation at K4 and K27 in the medial pre-frontal cortex (Black et al., 2006). The *c-fos* promoter in the striatum undergoes increased acetylation transiently following acute cocaine administration (Kumar et al., 2005). Chronic administration, however, hyperacetylates the brain derived neurotrophic factor (*Bdnf)* and *cdk5* promoters. These acetylation increases are mediated by DeltaFosB (Kumar et al., 2005). Chronic cocaine administration or stress reduces HDAC5 in the nucleus accumbens allowing for increased histone acetylation of target genes. Loss of HDAC5 causes sensitization to chronic cocaine and stress (Renthal et al., 2007). Mice lacking one allele of CBP [cAMP-response element binding protein (CREB) binding protein] have less accumulation of DeltaFosB. This is due to decreased acetylation at the FosB promoter. This makes them less sensitive to chronic cocaine when compared to wild type animals having both alleles of CBP (Levine et al., 2005).

CREB is a transcription factor critical in long-term memory formation (Yin et al., 1995). CREB activity is increased following psychostimulant drug administration such as cocaine and amphetamine (Hyman et al., 1995). These changes affect neural plasticity by affecting transcriptional regulation of downstream genes including the AMPA glutamate receptor subunit *GluR1*, brain derived neurotrophic factor (*Bdnf*) and tyrosine hydroxylase (Olson et al., 2005; Graham et al., 2007). CREB activation induces tolerance to the drug induced effects of cocaine by reducing the sensitivity of the drug. Overexpression of a dominant negative form of CREB enhances the sensitivity to cocaine. Therefore it is postulated that CREB functions in a feedback loop leading to a downregulation of dopaminergic reward mechanisms. This causes dysphoria leading to increased self administration and relapse (Carlezon et al., 1998).

Epigenetic modifications of the *slowpoke* promoter mediate drug tolerance

In Drosophila, transcriptional up-regulation of *slowpoke* following a single anesthetic dose of benzyl alcohol is mediated by a dynamic histone H4 acetylation pattern at conserved motifs in the control region of the gene (Wang et al., 2007).

We are interested in the mechanisms of rapid tolerance to abused drugs and volatile anesthetics in flies. The Ca^{2+}-activated K^{+} channel gene in flies encoded by the *slowpoke* locus is required for this tolerance. The gene is transcriptionally up-regulated following a single sedative dose of the drug leading to the observed behavioral tolerance (Ghezzi et al., 2004; Cowmeadow et al., 2006). Furthermore, we observed that message induction following benzyl alcohol sedation is caused by histone H4 acetylation at the promoter of *slowpoke* leading to neuronal induction of the gene and behavioral tolerance. This effect was phenocopied by administering flies the HDAC inhibitor, sodium butyrate. Furthermore, this acetylation is dependent on the Drosophila CREB transcription factor (*dCREB2*). The *slowpoke* promoter has conserved putative CREB binding sites (CRE sites). Occupancy of the *slowpoke* promoter by CREB was demonstrated by the ChromIP assay. The message induction following BA and behavioral tolerance was not seen in flies transgenically expressing a dominant negative form of CREB. The dominant negative CREB did not affect *slowpoke* message abundance relative to controls and only prevented the drug induced message up-regulation (Wang et al., 2007).

Tolerance to ethanol sedation is mediated by *slowpoke* induction (Cowmeadow et al., 2006). I used the ChromIP assay to detect changes in the histone H4 acetylation state of the promoter following a brief ethanol sedation. An increase in acetylation would be a direct correlate of message induction that causes behavioral tolerance in flies.

CHARACTERIZING BRAIN STRUCTURES INVOLVED IN TOLERANCE

The GAL4-UAS conditional gene expression system

Understanding the neuronal basis of ethanol tolerance requires characterization of the associated pathways and underlying brain regions mediating this phenotype. The requirement of the neuronal isoform of *slowpoke* in mediating rapid tolerance prompted us to inquire which regions of the fly brain are critical for *slowpoke* mediated tolerance. We employed the GAL4/UAS conditional gene expression system to drive a *slowpoke* cDNA in different regions of the adult fly brain. The availability of a large number of p[GAL4] lines from stock centers enabled us to assay important regions including neurotransmitter systems and centers involved in learning and memory in the fly.

Cocaine, a potent psychomotor stimulant, affects the dopamine transporter in the nucleus accumbens and increases dopamine levels. This causes increased locomotor effects and other positive reinforcing effects (Nestler, 2005). Similarly, in flies, cocaine induces psychomotor stimulation in a dose dependent manner and causes sensitization on repeated administration (Bainton et al., 2000; McClung and Hirsh, 1998). This behavior is analogous to that seen in higher mammals. Experiments performed where dopamine levels were reduced by administration of 3-iodotyrosine (3IY), show a reduction in the sensitization to cocaine induced locomotor activity (Bainton et al., 2000). Cocaine sensitive dopamine and serotonin transporters have been identified in flies (Corey et al., 1994; Porzgen et al., 2001; Demchyshyn et al., 1994).

Circadian genes modulate drug responses in both mammals and flies. Drosophila which have mutations in *per*, *cycle*, *Clock* or *doubletime* do not sensitize to cocaine (Andretic et al., 1999). Mice which lack a functional *per1* gene show decreased

behavioral responses to cocaine (Liu et al., 2005).

Another conserved pathway regulating ethanol sensitivity is the NEUROPEPTIDE Y (NPY) pathway. NPY and its cognate receptors mediate ethanol sensitivity in mammals, *C. elegans* and in flies (Thiele et al., 2004; Wen et al., 2005; Davies et al., 2004).

Nicotine is another drug which has conserved pathways in both flies and mammals. It binds to nicotinic acetylcholine receptors (nAChRs) that are widely distributed in the VTA and nucleus accumbens (Picciotto et al., 1998). The dopaminergic mesolimbic pathways have been implicated in causing the positive reinforcement effects of nicotine (Picciotto et al., 1998; Di Chiara, 2000). Mice lacking β2 subunit of nACHR do not show nicotine induced dopamine release in the nucleus accumbens (Picciotto et al., 1997). *C. elegans* show nicotine tolerance which is dependent on PKC signaling (Waggoner et al., 2000). In flies nicotine and cocaine act synergistically on dopaminergic pathways (Bainton et al., 2000). Thus, we see that conserved pathways regulate nicotine behaviors.

Genetic studies in flies have isolated cAMP/PKA pathway mutants which have altered ethanol sensitivity and tolerance. In flies, an allele of the *amnesiac* gene, *cheapdate* shows increased sensitivity to ethanol (Moore et al., 1998). A mutant for the Ca^{2+}/calmodulin-sensitive adenylate cyclase, *rutabaga* and mutants for the catalytic subunit of cAMP-dependent protein kinase (*pka*-C1) show increased ethanol sensitivity (Wolf and Heberlein, 2003). Conversely, a mutation in pka-RII gene encoding a regulatory subunit of PKA shows decreased ethanol sensitivity (Park et al., 2000). In mice, the cAMP pathway is important for ethanol sensitivity (Thiele et al., 2000). It has also been observed that ethanol increases dopamine signaling by modulating the

endogenous opioid receptors in rats (Gonzales and Weiss, 1998).

These observations reveal the remarkable similarity in the underlying neuronal pathways implicated in the actions of drugs of abuse in invertebrates and vertebrates. In mammals, the limbic circuits comprising the nucleus accumbens, ventral tegmental area, amygdala and hippocampus are all implicated in the reinforcing and reward pathways mediating drug addiction (Hyman et al., 2006). Conserved neurotransmitter systems in flies are shown to be regulated by drugs such as cocaine, nicotine and ethanol (Bainton et al., 2000). The mushroom bodies of the central brain in flies are the seat of olfactory learning and memory and regulate courtship conditioning, sleep and cAMP signaling (McGuire et al., 2001; Joiner et al., 2006; Waddell et al., 2000).

The neuronal isoform of s*lowpoke* is induced after a single dose of ethanol and is critical for ethanol tolerance in flies. To elucidate the spatial basis of this tolerance in the brain I used a GAL4/UAS inducible system to drive a UAS-*slowpoke* cDNA in different parts of the brain. This would point out important structures where tolerance is initiated. More importantly such a study would help characterize drug associated brain structures and engender characterization of the underlying molecular pathways. Furthermore critical genes regulating those pathways can be studied with respect to their epistatic relationship with *slowpoke* in mediating ethanol tolerance.

SYNAPTIC MECHANISMS OF ETHANOL TOLERANCE IN THE NERVOUS SYSTEM OF THE FRUIT FLY

Is tolerance an emergent or cell autonomous property of the nervous system?

The behavioral effects of acute ethanol administration in flies mirrors the behaviors we see in higher mammals. As soon as flies come in contact with ethanol they become very hyperactive and with continued contact progressively lose postural control characterized by the loss of righting reflex (LORR). Finally, they stop moving and become completely sedated. These phenotypes are comparable to those observed in higher vertebrates that start with loss of postural control and hyperactivity progressively leading to sedation upon continued exposure to the drug. What is remarkable is that ethanol sedation does not cause lethality in flies and they exhibit complete recovery and show rapid tolerance to sedation when assayed as early as 4-6 hours after a prior dose. We have also shown that this tolerance is pharmacodynamic and involves neuronal *slowpoke* function (Cowmeadow et al., 2005; Cowmeadow et al., 2006). Tolerance may be an emergent property of the nervous system or a cell autonomous property of individual neurons. If a blockade in neuronal signaling also blocks the acquisition of tolerance then this would indicate that tolerance is an emergent property of the nervous system that requires synaptic communication between different brain regions. If acquisition of tolerance is not blocked by reduction in neuronal signaling that indicates tolerance is a cell autonomous property of the brain that is independent of synaptic communication. To determine whether acquisition of tolerance requires neuronal communication I reversibly blocked neuronal signaling in the brain and then assayed for ethanol tolerance.

The temperature sensitive alleles shi^{ts2}, para^{ts1} and comt^{tp7}

I used three temperature sensitive mutants shi^{ts2}, para^{ts1}, comt^{tp7} to block synaptic transmission in the nervous system of the fly.

The allele *shi*^{ts2} (*shibire*) is the Drosophila homolog of dynamin and is required for synaptic vesicle endocytosis and recycling in the nervous system (Grigliatti et al., 1973; Koenig et al., 1983). At the restrictive temperature (30 ºC) there is an arrest of vesicle endocytosis. This leads to a rapid block in synaptic transmission.

The allele *para*^{ts1} (*paralytic*) is a mutation in the α-subunit of the voltage gated sodium channel gene in the fly nervous system. The *para*^{ts1} allele is a temperature sensitive paralytic mutation. At the restrictive temperature (30 ºC) the mutation blocks the propagation of neuronal action potentials and thereby blocks evoked vesicle fusion at the synapse causing a blockade of synaptic transmission (Suzuki et al., 1971; Siddiqi and Benzer, 1976; Loughney et al., 1989).

The allele *comt*^{tp7} (*comatose*) is a temperature sensitive mutant allele of the Drosophila NSF (N- ethylmaleimide- sensitive fusion factor) protein (dNSF1). The dNSF1 protein is involved in vesicular transport and synaptic vesicle exocytosis in the fly nervous system. The ATPase activity of NSF is thought to prime vesicles and promote docking and fusion of the vesicles at the pre-synaptic membrane. The *comt*^{tp7} allele in Drosophila is a temperature sensitive allele. At the restrictive temperature (35 ºC) the *comp*^{tp7} mutation blocks neurotransmitter release leading to a inhibition of synaptic transmission (Siddiqi and Benzer, 1976; Ordway et al., 1994).

The temperature sensitive paralysis due to the three mutant alleles is reversible and does not cause any long term changes in behavior or affect life span. These mutants

were used to induce a reversible paralysis following ethanol administration. I blocked synaptic neurotransmission immediately post ethanol administration for a period of 5 hours (*shi*^{ts2} and *para*^{ts1}) and 30 minutes (*comt*^{tp7}). I used a shorter time period for the *comatose* mutant flies as the recovery from paralysis is more prolonged than with the other two mutant alleles (Sanyal et al., 1999; Sanyal et al., 2001). The flies were then allowed to recover at room temperature overnight and tested for tolerance on the second day.

Temperature sensitive mutants of the shibire gene do not acquire tolerance

The Drosophila *shibire* gene was first isolated as a temperature sensitive paralytic mutation in the early 70's (Grigliatti et al., 1973). It was later identified to be the Drosophila homolog of *dynamin* (van der Bliek and Meyerowitz, 1991; Chen et al., 1991). The *shibire* allele *shi*^{ts2} shows a rapid paralytic phenotype at the restrictive temperature of 30 ºC due to a lack of vesicle recycling at the synapse and inhibition of synaptic transmission. The paralysis is reversible and flies recover very quickly when moved to the permissive room temperature.

I used *shi*^{ts2} flies to test whether functional ethanol tolerance is an emergent or cell autonomous property of the nervous system. I observed that a blockade of neuronal communication in these flies reduced the magnitude of ethanol tolerance. However this contrasted with the results seen with both *para*^{ts1} and *comt*^{tp7} as these mutant alleles did not block tolerance. Upon testing a different *shibire* allele, *shi*^{ts1}, I observed that flies did not acquire tolerance even at permissive temperatures. In light of these observations we believe that the *shibire* gene is involved in functional ethanol tolerance in flies.

Functions of shibire

The *shibire* locus in Drosophila encodes the dynamin protein, which is an

endocytic GTPase involved in vesicle recycling at the synapse. *Dynamin* was initially isolated from bovine brain tissue and subsequently the cDNA encoding the full rat brain isoform of *dynamin* was isolated (Shpetner and Vallee, 1989; Obar et al., 1990). The characterization of the *shibire* locus in Drosophila and its homology to *dynamin* proved to be a major factor in understanding the function of this gene (van der Bliek and Meyerowitz, 1991; Chen et al., 1991). Critical electrophysiological experiments paved the way for understanding how *shibire* encoded DYNAMIN regulates synaptic vesicle endocytosis at the synaptic membrane (Koenig et al., 1983). It also seems to selectively affect the endocytic component of synaptic transmission without perturbing exocytosis.

The mammalian *dynamin* genes have been classified into three groups; *dynamin* I that is neuronally expressed, a more ubiquitously expressed *dynamin* II and *dynamin* III that was first isolated from testis and now has been identified in the lung and brain. DYNAMIN participates in mechanochemical scission events at the synapse and the mechanics of its action are postulated to be a "pinching off" of vesicles whereas some have suggested it functions to "pop" them out. Nevertheless it is an important regulator of clathrin coated vesicle (CCV) recycling at the synapse (Stowell et al., 1999; Mears et al., 2007; Sweitzer and Hinshaw, 1998). In addition, the dynamins have also been implicated in non-clathrin vesicle budding such as caveolae budding, phagocytosis, golgi transport vesicle formation and regulation of actin assembly. Structurally, the classical dynamins have functionally important domains, the GTPase domain involved in membrane scission activities, middle domain and GED (GTPase effector domain) that mediates oligomerization events and regulates GTPase function (Marks et al., 2001). The PH (pleckstrin homology) domain plays an important role in endocytosis via its interactions with phosphoinositides in the membrane (Vallis et al., 1999). Another important domain

is the PRD (proline rich domain) and this is important for SH3 type interactions with numerous other proteins functioning at the synapse including AMPHIPHYSIN, ENDOPHILIN and signaling molecules and scaffolding proteins such as SHANK (Schmid et al., 1998; Solomaha et al., 2005; Okamoto et al., 2001). The PH domain and PRD function to target *dynamin* to the correct subcellular locations via protein-lipid and protein-protein interactions.

The *shibire* locus in Drosophila has been fundamental in the characterization of DYNAMIN as a regulator of synaptic vesicle endocytosis. The temperature sensitive alleles of the gene (ts alleles) exhibit a reversible blockade of synaptic transmission at the restrictive temperature of 29 ºC to 38 ºC leading to rapid paralysis. The excitatory junction potential at the synapse is dramatically reduced and there is a depletion of synaptic vesicles at the restrictive temperature. Membrane invaginations can be visualized as "collared pits" which appear arrested because they are unable to be endocytosed thus emphasizing the role of *shibire* in synaptic vesicle recycling (Koenig et al., 1983; Zhang, 2003).

A synaptic signaling complex mediating drug responses in mammals

One of the preferred targets of ethanol in the brain is glutamatergic transmission. Ethanol is known to inhibit NMDA receptors (Lovinger et al., 1989; Wirkner et al., 1999; Narahashi et al., 2001). The scaffolding protein SHANK helps to organize NMDA receptors at the post-synaptic density (PSD) by recruiting additional proteins such as PSD-95 and HOMER. These interactions regulate the morphology and maturation of dendritic spines (Sala et al., 2001; Kim and Sheng, 2004; Xiao et al., 2000; Naisbitt et al., 1999). The EVH1 domain of homer has a strong binding affinity to poly-proline motifs (PPXXF) present in SHANK, mGluR's, inositol triphosphate receptors (IP3R's) and

ryanodine receptors (RyR's) (Beneken et al., 2000). SHANK is a component of PSD-95/ GKAP/NMDA receptor complex (Naisbitt et al., 1999). This establishes a biochemical link between HOMER associated synaptic proteins and the NMDA receptor complex (Tu et al., 1999).

Drug responses in mammals and flies are regulated by homer

The synaptic gene *homer* has been implicated in the sensitivity to the acute and chronic effects of cocaine administration and in mediating behavioral neuroplasticity to ethanol and regulating its effects on dopaminergic and glutamatergic transmission in mice (Szumlinski et al., 2004; Szumlinski et al., 2005). The mammalian DYNAMINS participate in a lot of protein-protein interactions via SH3 interactions in the PRD (proline rich domains). Proteins such as phospholipase Cγ, phosphatidyl-inositol-3-kinase, mixed-lineage kinase 2 interact via SH3 domains with DYNAMIN (Gout et al., 1993; Solomaha et al., 2005; Rasmussen et al., 1998). Interestingly, in mammalian systems DYNAMIN III has been shown to interact at the PSD with HOMER via its EVH1 domain (Gray et al., 2003).

Flies have only one DYNAMIN encoded by the *shibire* locus and it is possible that it could interact with Drosophila HOMER and influence drug induced neuronal plasticity at the synapse. Drosophila has single genes encoding for *homer* and the *shank* homolog (*prosap*). In flies, HOMER and PROSAP have been shown to directly interact similar to their mammalian counterparts (Diagana et al., 2002). Drosophila HOMER has 73% homology to the mammalian homologs and is targeted to the ER and dendrites. Also, flies mutant for *homer* show behavioral defects in locomotor activity and behavioral plasticity (Diagana et al., 2002).

Drosophila *homer* has been implicated in ethanol tolerance (Urizar et al., 2007).

Null mutants for *homer* show an increased sensitivity and decreased tolerance to the sedative effects of ethanol. Although Drosophila DYNAMIN does not have a conserved HOMER binding motif (PPXXF), there is a SHANK homolog in Drosophila, the gene *prosap*. PROSAP has conserved SH3 and pdz domains that participate in protein-protein interactions and could probably interact with the proline rich region of DYNAMIN (Figure 5.6). Mammalian SHANK has been shown to bind HOMER and help in clustering and organization of NMDA and metabotropic receptor pathways (Tu et al., 1999). There is a conserved HOMER binding motif in Drosophila PROSAP (Figure 5.6). This suggests that PROSAP is an ideal candidate to function as an adapter protein and recruit HOMER and DYNAMIN thereby forming a signaling complex at the synapse. This complex could play an important role in mediating ethanol tolerance in flies.

Chapter 2: Post-translational modifications of the BK channel in Drosophila mediate acute ethanol tolerance

INTRODUCTION

Tolerance is "a reduction in drug sensitivity caused by prior exposure". There are different kinds of tolerance; acute, rapid and chronic (Cowmeadow et al., 2005; Berger et al., 2004). Tolerance can also be classified as pharmacodynamic or functional (due to neuronal adaptation at the cellular level) and pharmacokinetic (due to a change in alcohol metabolism) tolerance. We are interested in understanding the molecular mechanisms of rapid functional tolerance to ethanol in Drosophila.

Drosophila is a good model system to study ethanol behaviors

Drosophila has been a good model system to study the acute effects of ethanol sedation (Cowmeadow et al., 2005; Singh and Heberlein, 2000). Flies exhibit similar phenotypic effects to acute ethanol administration as higher vertebrates such as initial hyper-activity that is followed by loss of coordination and sedation. Flies are very amenable to genetic manipulation and can be used for high throughput behavioral screens. They have been shown to acquire rapid pharmacodynamic or functional tolerance to the sedative effects of ethanol using different behavioral assays (Scholz et al., 2000; Cowmeadow et al., 2005; Ramazani et al., 2007).

A number of candidate genes that affect ethanol behaviors have been isolated in flies. The *cheapdate* (*chpd*) allele, a transposon-induced mutation of the *amnesiac* gene (*amn*), regulates critical cellular pathways such as cAMP signaling and displays increased sensitivity to ethanol induced loss of postural control (Moore et al., 1998). Genetic screens for ethanol behaviors have also identified more candidates such as *barfly* (*brf*)

and *tipsy* (*tps*) that show reduced and increased ethanol sensitivity respectively (Singh and Heberlein, 2000). However, the molecular functions of *brf* and *tps* are yet to be characterized. Pathways involving stress responses have also been implicated in ethanol tolerance in Drosophila (Scholz et al., 2000; Scholz et al., 2005). Similarly NEUROPEPTIDE F (NPF) and its receptor NPFR1 have been implicated in acute sensitivity to ethanol sedation with disruptions of the pathway resulting in reduced sensitivity (Wen et al., 2005). NPF is the Drosophila homolog of mammalian NEUROPEPTIDE Y (NPY) which has been shown to have similar effects to ethanol in higher vertebrates (Thiele et al., 2004). Studies have shown that changes in the actin cytoskeleton in flies affect ethanol responses in flies and mice (Rothenfluh et al., 2006; Offenhauser et al., 2006).

Rapid tolerance to anesthetics and ethanol is mediated by a transcriptional up-regulation of *slowpoke*

We have been interested in the transcriptional regulation of the *slowpoke* (BK channel) gene in Drosophila in response to volatile anesthetics such as benzyl alcohol (BA) and abused drugs such as ethanol. Using the "loss of righting reflex" assay (LORR) we have shown the *slowpoke* gene in flies is responsible for the acquisition of rapid pharmacodynamic tolerance to benzyl alcohol and ethanol sedation. The evidence that tolerance to sedation by BA and ethanol is mediated by a transcriptional up-regulation of *slowpoke* is substantial. The neuronal isoform of the gene is transcriptionally up-regulated following an acute exposure to both drugs. Flies that carry null mutations in *slowpoke* (slo^4) are unable to acquire rapid tolerance to both drugs. Transgenic induction of a *slowpoke* cDNA in slo^4 flies confers BA and ethanol resistance. Furthermore, the histone deacetylase inhibitor, sodium butyrate, that stimulates *slowpoke* transcription

because of its effects on histone acetylation, also produces *slowpoke* dependent behavioral resistance to BA sedation (Ghezzi et al., 2004; Cowmeadow et al., 2005; Cowmeadow et al., 2006; Wang et al., 2007).

Furthermore, the transcriptional up-regulation in response to BA sedation is mediated by a dynamic histone H4 acetylation change at the promoter regulated by the Drosophila CREB transcription factor. The CREB transcription factor is shown to occupy the *slowpoke* promoter and regulates *slowpoke* expression. BA sedation enhances the activity of CREB-mediated transcription. A mutation in CREB that prevents BA-induced *slowpoke* up-regulation also prevents the acquisition of tolerance (Wang et al., 2007). Furthermore, expression of a CREB transgene that is believed to be constitutively active phenocopies BA tolerance (*From Yan Wang's Dissertation 2007*).

Can post-translational modifications to the BK channel in flies cause ethanol tolerance?

While the evidence for a role of transcriptional regulation of BK channel expression in the production of tolerance in flies is substantial, this should not be taken to mean that the channel activity is not being directly regulated by anesthetic or alcohol sedation. Important cellular events are typically regulated at multiple levels. It is likely that, in response to a single environmental stimulus, *slowpoke* expression is regulated at the level of transcription, mRNA splicing and perhaps mRNA editing while the activity of the protein is regulated at the level of phosphorylation, association with accessory subunits and membrane localization.

In mammalian systems, studies have focused on the post-translational regulation of BK channels and not on transcriptional regulation of the gene in response to alcohol exposure. It has been clearly shown that BK channels are a direct target of ethanol

(Brodie et al., 2007). The core-linker region of the mouse BK channel, MSLO (S0-S8) has been shown to be critical for ethanol mediated activation of the channel (Liu et al., 2003). BK channels are regulated by protein kinases including PKA, PKG and PKC and have multiple phosphorylation sites in the carboxy-terminal "tail" (Schubert and Nelson, 2001). Ethanol potentiation of BK channels in pituitary GH3 cells is due to PKC-mediated phosphorylation, with PKC inhibitors abolishing this effect (Jakab et al., 1997). Furthermore, in slice, explant and tissue culture it has been shown that physiological correlates of ethanol tolerance involve phosphorylation-mediated changes in BK channel activity and even ethanol induced redistribution of BK channels in the cell membrane (Liu et al., 2006; Pietrzykowski et al., 2004). CAMKII phosphorylation of a threonine residue, Thr107 in the S0-S1 loop functions to inhibit ethanol's effects on the BSLO channel (bovine BK). Dephosphorylation of Thr107 in BSLO by CAMKII inhibitors reverses this effect and causes ethanol potentiation of the channel. This residue is not conserved and substitution by a valine that is insensitive to phosphorylation in MSLO and HSLO (human BK) channels causes ethanol activation. The CAMKII phosphorylation of Thr107 in BSLO has been implicated in ethanol induced plasticity ranging from ethanol activation in dephosphorylated states to inhibition when all four subunits are phosphorylated (Liu et al., 2006). Thus, it is likely that in flies ethanol exposure also produces post-translational modifications that alter channel activity and even perhaps result in redistribution of the channel in the cell membrane.

We have already shown that volatile anesthetics and abused drugs such as benzyl alcohol (BA) and ethanol act via transcriptional induction of *slowpoke* to mediate rapid functional tolerance (Ghezzi et al., 2004; Cowmeadow et al., 2005; Cowmeadow et al., 2006; Wang et al., 2007). Furthermore, with BA we observed that this induction is

dependent on increased histone H4 acetylation at conserved regions in the promoter of the gene. This is mediated by the activity of the CREB transcription factor. Flies that are mutant for CREB are not able to acquire tolerance and do not show the drug induced message up-regulation, although the message abundance is normal compared to control flies. Transgenic expression of a CREB activator mimics tolerance (From *Yan Wang's Dissertation 2007*). Additionally treatment with the HDAC inhibitor, sodium butyrate, seems to phenocopy message induction and confer BA resistance. The above evidence strongly suggest that a transcriptional regulation of *slowpoke* is necessary for rapid tolerance in flies. One way to determine whether ethanol exposure post-transcriptionally regulates the gene is to determine whether a transcriptionally uninducible *slowpoke* transgene can support the acquisition of ethanol tolerance. If this occurs, it would be clear evidence of additional levels of regulation downstream of the act of transcription.

The B52H transgene, while temperature inducible, constitutively expresses a single splice variant of slowpoke. I used heat induction of B52H to show that increased expression of *slowpoke* is sufficient to produce an ethanol resistance phenotype (a phenocopy of tolerance) (Cowmeadow et al., 2006). I also show that B52H constitutively expresses *slowpoke* at low levels (uninduced expression from the heat shock promoter) and that ethanol sedation does not increase B52H mRNA expression levels. These animals therefore carry a *slowpoke* gene that is not ethanol inducible and can therefore be used to examine the post-transcriptional effects of ethanol on the gene. A limitation of this experiment is that the B52H transgene expresses only a single *slowpoke* splice variant whereas the endogenous gene can express almost 300 isoforms. Importantly, however, I show that the constitutively expressed B52H transcript can restore the capacity to acquire tolerance to animals homozygous for the slo^4 null mutation. These

results suggest that there is an independent post-transcriptional regulation of the gene by ethanol.

RESULTS

Transgenic induction of a *slowpoke* cDNA phenocopies rapid ethanol tolerance.

We have shown that rapid ethanol tolerance in flies is due to neuronally expressed *slowpoke*. Flies which are homozygous null for the gene (slo^4) do not acquire tolerance (Cowmeadow et al., 2005; Cowmeadow et al., 2006). To see if artificial induction of *slowpoke* in a mutant background restores resistance to ethanol sedation, I used the B52H transgenic flies that carry a heat shock inducible *slowpoke* cDNA in a slo^4 background.

Age matched B52H flies were divided into two groups, one that received air (Control) and the other that received a heat pulse at 37 °C for 30 minutes (HS). The flies were assayed for ethanol tolerance at two time intervals, 6 hours after the heat shock (Figure 2.1A) and 24 hours after the heat shock (Figure 2.1B). Two time points were assayed to mirror the tolerance assays done with wild type (CS) flies (Cowmeadow et al., 2005). The two experiments were done with age matched controls which received air. Figure 2.1A shows the results 6 hours after a heat shock induction of the transgene. Heat shock induction of the transgene (HS) causes resistance to ethanol sedation when compared to non heat shocked (Control) flies as measured by recovery from sedation by return of postural control. Figure 2.1B shows the same results 24 hours after the heat shock induction. Figures 2.1C and 2.1D are control experiments performed on wild type Canton S (CS) flies subjected to the same protocol to show that there is no effect of heat on ethanol tolerance.

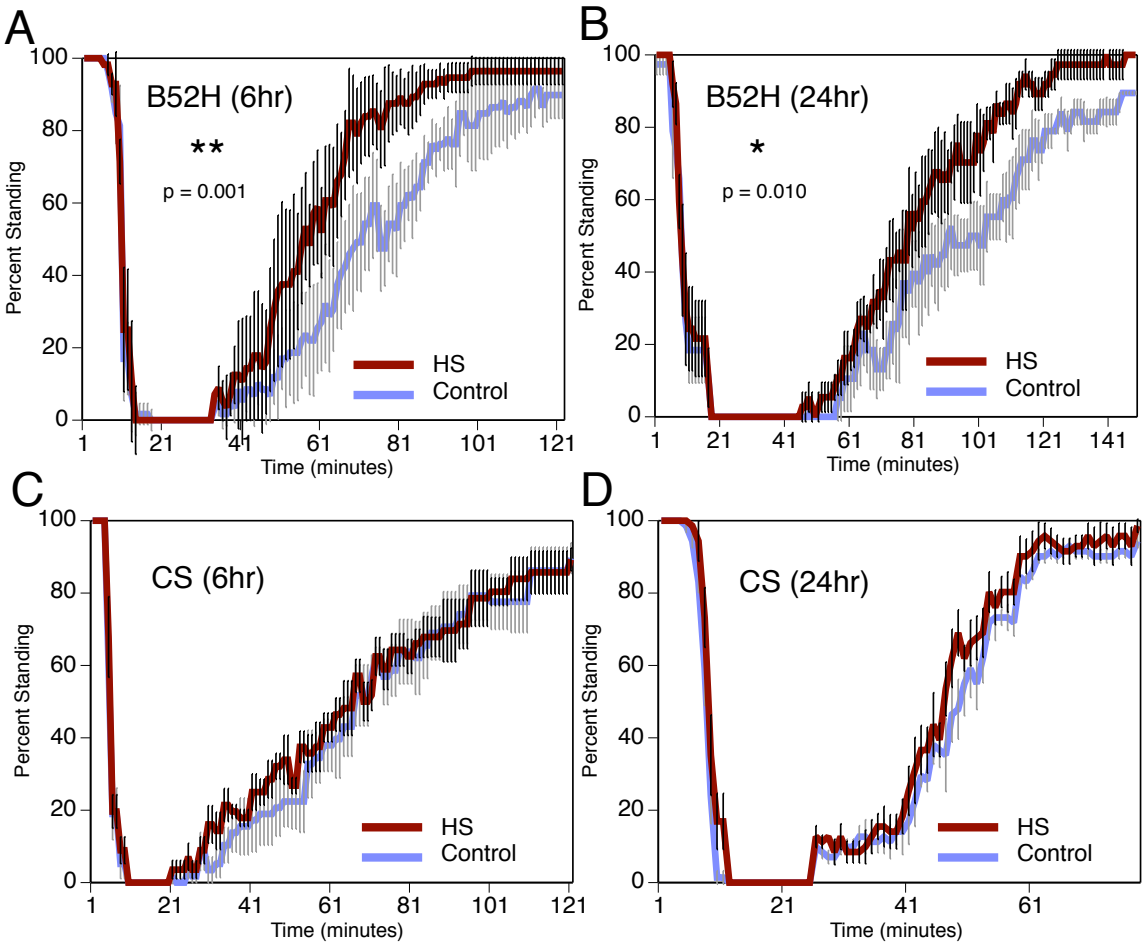


Figure 2.1. Transgenic induction of *slowpoke* phenocopies ethanol tolerance. **A)** B52H flies were age matched and divided into two groups. control flies received air and the heat shock group received a 37 °C incubation for 30 minutes (HS). After 6 hours a tolerance assay was performed where both groups received ethanol and were subjected to the tolerance assay. Recovery assayed after switching the ethanol stream to air and counting the number of flies which regained postural control and recovery of the righting reflex. We see that heat induction of *slowpoke* mimics tolerance after 6 hours in B52H flies. **B)** B52H flies were subjected to the same protocol as in 2.1A, Control and HS but here I waited 24 hours to do the tolerance assay. Recovery assayed after switching the ethanol stream to air and counting the number of flies which regained postural control and exhibited the righting reflex. We observed that heat induction of *slowpoke* mimics tolerance after 24 hours in B52H flies. **C** and **D** are controls done with wild-type flies, Canton S (CS) which show that there are no effects of heat on ethanol tolerance and the tolerance we see in

B52H is due to *slowpoke* induction.

Statistical analysis for all the graphs is by the log rank test. This accounts for the differences in recovery of the whole population between the two curves (*$p<0.05$; **$p<0.01$). The p-values are indicated on the graphs if significant. Error bars are SEM for each data point.

The data above (Figure 2.1) indicate that transgenic *slowpoke* induction in mutant flies (slo^4) is able to phenocopy or mimic the ethanol tolerance observed in wild type flies. We had previously shown that ethanol administration to wild type flies causes an up-regulation of the neuronal isoform of the endogenous *slowpoke* gene that is necessary for acquisition of rapid tolerance. Flies which are homozygous null for *slowpoke* (slo^4) do not acquire tolerance. The B52H resistance assay (Figure 2.1) consolidates these observations and illustrates the importance of *slowpoke* message up-regulation in mediating tolerance. I have shown that ethanol resistance can be restored in slo^4 flies by artificially inducing a *slowpoke* cDNA.

Rapid ethanol tolerance is independent of *slowpoke* transcription - probably a post-translational effect.

We have substantial evidence that shows that both the solvent anesthetic benzyl alcohol and ethanol transcriptionally induce *slowpoke* and this induction is critical for the acquisition of tolerance. The neuronal isoform of the gene is transcriptionally up-regulated following an acute exposure to both drugs. Flies which carry null mutations in *slowpoke* (slo^4) are unable to acquire rapid tolerance to both drugs (Ghezzi et al., 2004; Cowmeadow et al., 2005; Cowmeadow et al., 2006). Transgenic induction of a *slowpoke* cDNA in slo^4 flies confers BA (Ghezzi et al., 2004) and ethanol (Figure 2.1) resistance. Following administration of the HDAC inhibitor, sodium butyrate, *slowpoke* transcription is induced phenocopying tolerance (Wang et al., 2007).

Furthermore, the transcriptional induction in BA treated flies is dependent on the CREB transcription factor. BA sedation induces CREB occupancy at conserved CRE motifs in the *slowpoke* promoter. Activation of a dominant negative CREB transgene reduces BA sedation-induced *slowpoke* expression and thereby preventing the acquisition of tolerance (Wang et al., 2007).

Critical cellular pathways are typically regulated at multiple levels. It is likely that, in response to a single drug sedation, *slowpoke* is regulated at the level of transcription, mRNA splicing and perhaps mRNA editing and the activity of the protein is regulated via phosphorylation, association with accessory subunits and membrane localization.

In mammalian systems some of the well characterized drug induced changes in BK channel activity are due to post-translational modifications (Brodie et al., 2007; Pietrzykowski et al., 2004). Therefore it is possible that in flies, ethanol can alter

SLOWPOKE channel activity by post-translational modifications in addition to the observed transcriptional regulation.

To identify a transcription independent mechanism of *slowpoke* regulation, I needed a *slowpoke* gene that is not inducible by ethanol. Then the tolerance observed could be attributed to a transcription independent, possible post-translational effect on the channel. I used the B52H transgene to test this idea. Without heat induction, the expression from the transgene is very minimal and if these flies were able to acquire tolerance then it would indicate a transcription independent mechanism.

Heat shock promoters (HSP70) are known to be have "leaky" expression at room temperature. Since B52H carries a HSP70 driving a *slowpoke* cDNA we can assume that a minimal amount of *slowpoke* mRNA is always present in the fly brain. To test if *slowpoke* has a transcription independent mechanism mediating ethanol tolerance, I performed a tolerance assay on uninduced B52H flies where control flies that received air (Control) on day 1 were compared to flies that received an ethanol dose on day 1 (Ethanol). On day 2, both groups received ethanol and were subjected to the tolerance assay. Recovery from sedation was assayed by return of postural control (righting reflex). I observed that B52H flies were able to acquire tolerance after a prior sedative dose of ethanol (Figure 2.2A). This tolerance is significant and comparable to the wild-type tolerance observed with CS flies (Figure 2.2B).

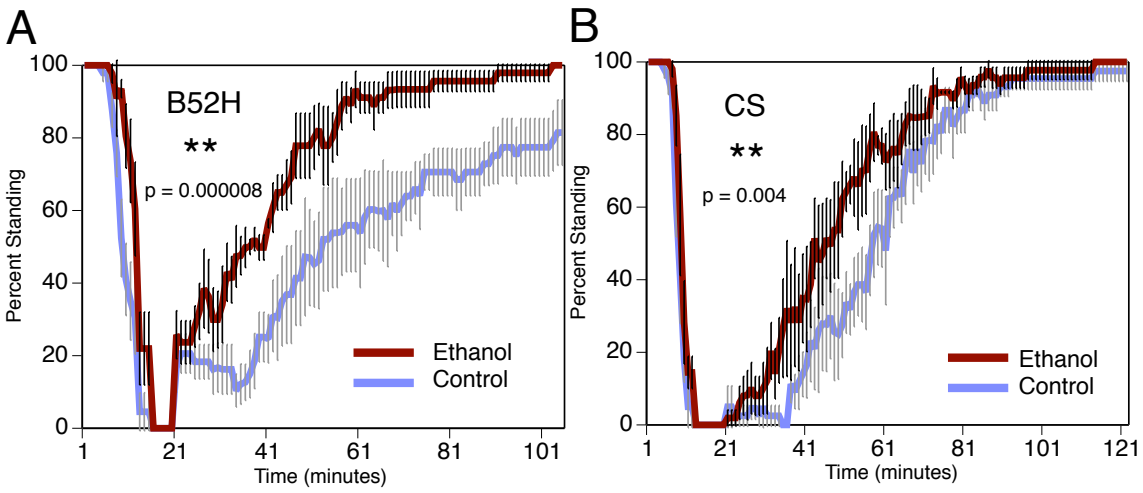


Figure 2.2. Ethanol tolerance is independent of *slowpoke* transcription - Uninduced B52H flies can acquire tolerance. **A)** B52H female flies were age matched and split into two groups. One group received air (Control) on day 1 while the second group received a saturated stream of ethanol on day 1 (Ethanol). On day 2 both groups received ethanol and recovery from sedation assayed after switching the ethanol stream to air and counting the number of flies which regained postural control. We see that B52H flies can acquire tolerance without a heat shock induction of *slowpoke* cDNA on day 1. **B)** Canton S (CS) flies were subjected to the same treatment to show that wild-type flies can acquire tolerance.

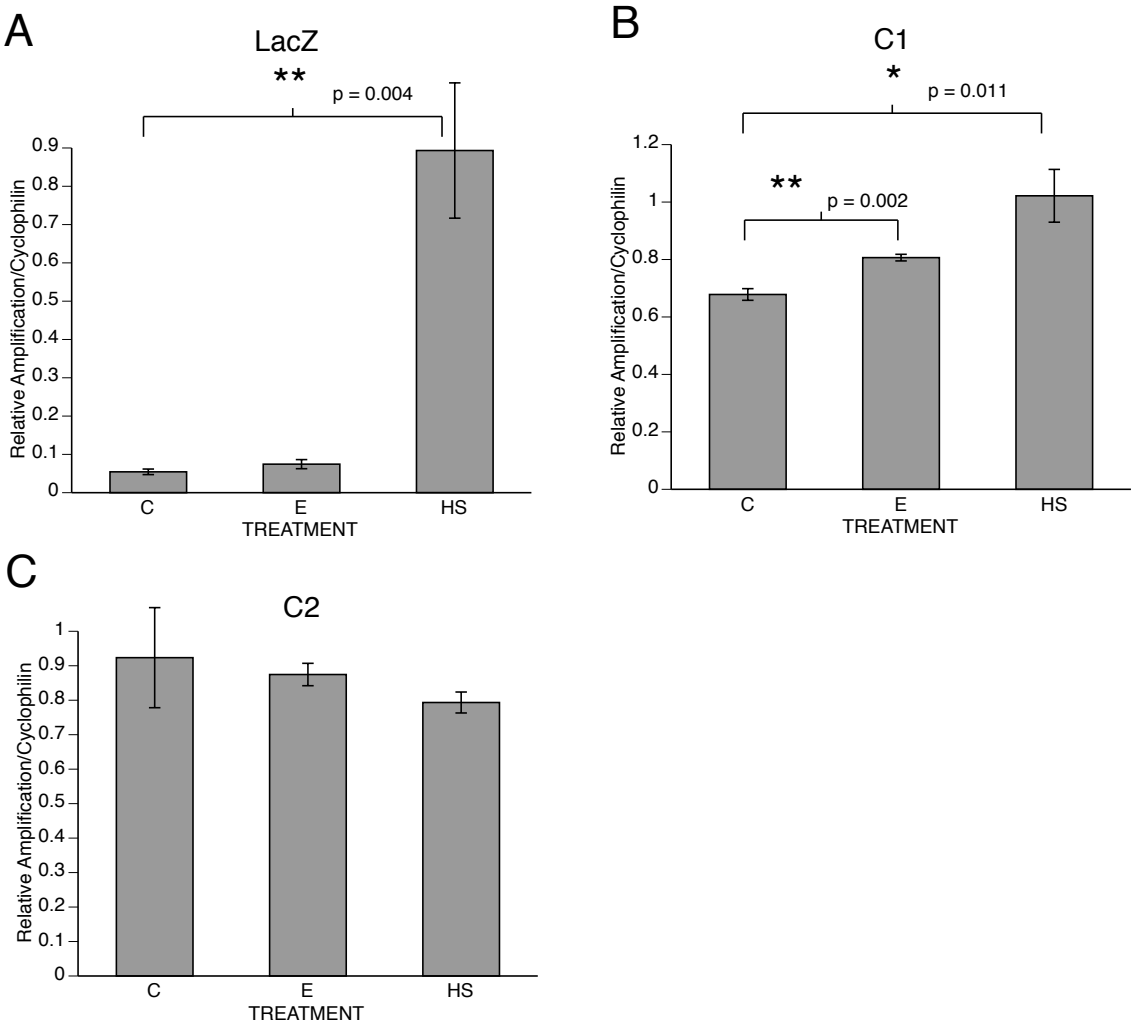
Statistical analysis is by the log rank test. This accounts for the differences in recovery of the whole population between the two curves (*p<0.05; **p<0.01). The p-values are indicated on the graphs if significant. Error bars are SEM for each data point.

An important question I had to address was whether ethanol could be inducing HSP70 and causing an up-regulation of *slowpoke* message levels. If it did, then the tolerance we observed (Figure 2.2A) could be due to the up-regulation of *slowpoke* cDNA by ethanol activating the heat shock promoter much akin to the heat shock induced up-regulation leading to resistance in these flies (Figure 2.1A and 2.1B). The B52H transgenic construct has a heat shock promoter which drives a *slowpoke* cDNA. It also contains a downstream *lacZ* (β-galactosidase) gene. Translation of the *slowpoke* message does not make a fusion protein with *lacZ* because of a UGA stop codon in the *slowpoke* sequence. However, this gives us a convenient way to measure the *lacZ* message because flies do not have an endogenous *lacZ* gene (Figure 2.3D). The amount of *lacZ* message is a direct 1:1 correlate of the amount of *slowpoke* induced by the heat shock promoter.

I made primers recognizing the lacZ portion of the transgene and measured message levels 6 hours after the three treatments; control (C), ethanol (E) and heat shock (HS). When I measured levels of the *lacZ* mRNA from B52H flies 6 hours after a single ethanol dose (E) I did not observe a significant message up-regulation compared to air treated controls (C) (Figure 2.3A). This contrasted with the very robust and significant up-regulation of *slowpoke* cDNA after a heat shock (HS) (Figure 2.3A).

To control for drug administration I needed a positive control that shows predictable induction following ethanol sedation. I used the endogenous slo^4 allele to address this issue. Even though B52H is in a slo^4 mutant background it carries the intact neuronal (C1) and muscle promoter (C2) from the endogenous gene. This enables us to measure the truncated *slowpoke* pre-mRNA encoded by these promoter driven exons. We had previously shown that C2 doesn't change while C1 shows a significant increase after ethanol sedation (Cowmeadow et al., 2006). So I measured the *slowpoke* C1 (neuronal)

and C2 (muscle) pre-mRNA 6 hours following ethanol sedation. The C2 primers recognize the portion of the exon exclusive from the B52H transgene so that we were measure from the endogenous pre-mRNA and not the transgenic cDNA. As expected, I observed an increase in C1 (Figure 2.3B) and no significant change in C2 (Figure 2.3C).



D

B52H Transgene

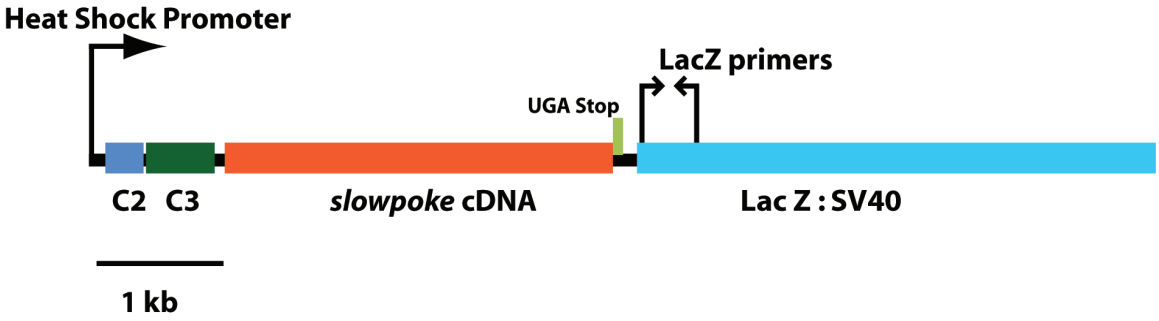


Figure 2.3. Ethanol sedation does not induce the heat shock promoter in B52H. Total RNA made from B52H flies was used in a reverse transcription reaction with oligo dT primers (Invitrogen®) and assayed by real time PCR for mRNA abundance relative to *cyclophilin*. The three treatment groups were air (C), ethanol sedation (E) and heat shock (HS). After 6 hours the flies were frozen and RNA extracted. **A)** shows the amount of lacZ message relative to *cyclophilin* and there is no induction in the flies which received ethanol (E) when compared to the 7-fold induction seen with heat shock (HS). **B)** shows the amount of endogenous neuronal C1 exon from the pre-mRNA relative to *cyclophilin*. As expected there is an increase in message abundance relative to controls in the ethanol sedated (E) group and we also see an induction in the heat shock group (HS). **C)** shows the relative amplification levels of the muscle exon C2 that doesn't change with ethanol treatment (E). The error bars are SEM. n=3 *p<0.05; **p<0.01 Student's t-test.
D) is a map of the B52H transgenic construct. It also indicates the primers used to amplify the lacZ message in 2.3A.

The above data (Figures 2.2 and 2.3) indicate that ethanol can also regulate *slowpoke* in a transcription independent manner to mediate tolerance. This could be a modulatory pathway in addition to the transcriptional up-regulation we observe following ethanol administration. The probable nature of this transcription independent mechanism could be post-translational as that is the most commonly observed mechanism in mammalian systems.

DISCUSSION

Tolerance to sedation with the anesthetic benzyl alcohol or with ethanol involves the transcriptional activation of the *slowpoke* gene (Ghezzi et al., 2004; Cowmeadow et al., 2006). The histone deacetylase inhibitor, sodium butyrate, produces *slowpoke* dependent behavioral resistance to BA sedation by stimulating *slowpoke* transcription and mimicking tolerance (Wang et al., 2007)

Additional evidence for a transcriptional role in the production of tolerance can be found in the study of the CREB transcription factor. CREB directly regulates *slowpoke* gene expression and CREB-mediated transcription is enhanced by benzyl alcohol sedation. Mutations in CREB prevent the acquisition of tolerance and also prevent BA-induced *slowpoke* induction (Wang et al., 2007). In addition, transgenic expression of a CREB activator transgene stimulates *slowpoke* expression and mimics tolerance (From *Yan Wang's Dissertation*). These results strongly suggest that a transcriptional regulation of *slowpoke* is important for drug tolerance in flies.

Important cellular pathways are typically regulated at multiple levels following a single environmental stimulus. In mammalian systems, studies have focused on the post-translational regulation of BK channels and not on transcriptional regulation of the gene

in response to alcohol exposure. It has been clearly shown that BK channels are a direct target of ethanol (Brodie et al., 2007). Ethanol effects on BK channels involve phosphorylation-mediated changes channel activity and in some instances ethanol induced redistribution of BK channels in the cell membrane (Liu et al., 2006; Pietrzykowski et al., 2004). Recent literature shows that ethanol's effects on BK channels can be modified by CAMKII phosphorylation. A specific residue in bovine SLOWPOKE channels (BSLO) has been shown to be phosphorylated by Ca^{2+}/calmodulin dependent protein kinase II (CAMKII) affecting channel activation and redistribution in the membrane following ethanol treatment (Liu et al., 2006). Thus, it is likely that in flies, that ethanol exposure also produces post-translational modifications that alter channel activity and even perhaps results in redistribution of the channel in the cell membrane.

One way to determine whether alcohol exposure post-transcriptionally regulates the activity or membrane localization of SLOWPOKE channel is to determine whether a transcriptionally uninducible *slowpoke* transgene can acquire alcohol tolerance. This would suggest that there are additional levels of regulation of *slowpoke* in response to ethanol.

The tolerance observed in uninduced B52H flies seems to suggest that ethanol tolerance can be acquired by transcription independent mechanisms. We postulate that this is post-translational based on evidence from the mammalian literature as that is the preferred mode of ethanol regulation of the gene. B52H flies express a *slowpoke* cDNA under the control of a heat-shock promoter (Atkinson et al., 1998). B52H flies are homozygous for the slo^4 mutant allele. This allele is a chromosomal inversion with one endpoint of the inversion located within the *slowpoke* gene. While the allele still expresses a truncated transcript, it is a null as confirmed both electrophysiologically and

by complementation assays. Therefore, the only source of functional *slowpoke* message in B52H flies is from the heat shock inducible transgene. The resistance assay indicates that B52H flies are able to acquire resistance to the sedative effects of ethanol following induction of the transgene by heat shock. Since the transgene is not inducible by ethanol these flies can also be used to examine post-transcriptional ethanol regulation. The tolerance assay reveals that B52H flies can acquire tolerance without induction of the transgene which suggests a transcription independent mechanism of tolerance. This is most likely a post-translational effect of ethanol on the channel.

The SLOWPOKE channel in Drosophila has numerous putative phosphorylation sites at both its N and C - termini. The channel has been shown to associate with Src kinases and the catalytic subunit of PKA (Wang et al., 1999). Although there are no β subunits for BK identified in Drosophila, the channel associates with accessory proteins such as slowpoke-binding protein (SLOB) and slowpoke-interacting protein (SLIP) which influence channel kinetics (Schopperle et al., 1998; Xia et al., 1998). SLOB is, in turn, phosphorylated by protein kinases and interacts with the scaffolding protein, 14-3-3 (Jaramillo et al., 2006; Zeng et al., 2004). We envision that ethanol either directly influences channel phosphorylation or indirectly impacts accessory SLOWPOKE binding proteins such as SLOB or 14-3-3. The endogenous gene in Drosophila has 5 tissue specific promoters and 14 alternatively spliced exons with different splice variants contributing to different channel kinetics (Lagrutta et al., 1994; Yu et al., 2006). Since B52H encodes for a single splice variant of *slowpoke* and these flies are able to acquire tolerance, we could identify the specific residues that are substrates for kinases and start characterizing them further. This is possible by deleting specific residues in the B52H sequence and making new transgenic flies that can then be assayed for the inability to

acquire ethanol tolerance. Another approach is by targeting the protein kinases such as PKC and CAMKII by using RNAi against the genes or toxins. This prevents phosphorylation of the channel and the resulting effects on ethanol tolerance can be assayed. These approaches can help identify a post-translational mechanism of ethanol regulation of SLOWPOKE.

I have shown that ethanol's actions on the *slowpoke* gene, apart from the observed transcriptional up-regulation, also influence post-translational modifications leading to rapid ethanol tolerance. This effect manifests as early as 6 hours post ethanol exposure and lasts until 24 hours. These modifications probably modify some aspect of the channel or a binding partner of the channel or even the surrounding milieu. If we are able to identify an ethanol responsive molecular pathway that regulates *slowpoke* post-translationally, it will be an important finding that emphasizes the significance of ethanol as a unique drug that regulates a gene both transcriptionally and post-translationally.

Chapter 3: Ethanol induced epigenetic modification of the BK channel gene promoter causes message induction

INTRODUCTION

Drosophila is a good model system to study acute tolerance to the sedative effects of volatile organic solvents and abused drugs such as benzyl alcohol and ethanol respectively. We have shown that this tolerance is neuronal in origin and is seen as early as 4-6 hours following a prior exposure to the drug. Tolerance to drug induced sedation has been shown to be dependent on an up-regulation of the *slowpoke* Ca^{2+}-activated K^{+} channel gene. Mutants for *slowpoke* do not acquire tolerance and transgenic induction of the cDNA in a null background confers resistance (Ghezzi et al., 2004; Cowmeadow et al., 2005; Cowmeadow et al., 2006).

Histone modifications mediate drug responses

One of the preliminary steps in the regulation of euchromatic genes is chromatin remodeling thereby making promoters accessible for transcription factor binding and RNA polymerase II complex assembly (Berger, 2007). Some of the well characterized post-translational modifications (PTMs) that mediate gene activation are acetylation (H3- 9, 14, 18, 56; H4- 5, 8,12,16; H2A and H2B) and methylation (H3- 4,36,79) at lysine residues. Methylation can also decrease transcription at different lysine residues. Histone acetylation can make the promoter more accessible for transcription factor binding and induce gene expression. Transcriptional activators and co-activators recruit HATs and in some cases like the circadian clock regulator, CLOCK, can have intrinsic HAT activity (Doi et al., 2006). These activities result in promoter accessibility to RNA polymerase II complexes and increased transcription from the locus. Such changes can lead to

regulation of gene expression and in the case of drug addiction when the stimulus is persistent lead to stable alterations in the neural circuits and neuronal plasticity. Cocaine administration in adolescent rats results in increased adult responses to cocaine. This persistent change has been shown to be accompanied by a decrease in histone H3 methylation at K4 and K27 in the medial pre-frontal cortex (Black et al., 2006). The *c-fos* promoter in the striatum has been shown to have increased acetylation transiently following acute cocaine administration (Kumar et al., 2005). Chronic administration hyperacetylates the brain derived neurotrophic factor (*Bdnf)* and *cdk5* promoters. These acetylation changes are induced by the transcription factor, DeltaFosB (Kumar et al., 2005). Chronic cocaine administration reduces HDAC5 in the nucleus accumbens thereby promoting increased histone acetylation of target genes. Loss of HDAC5 has been shown to cause sensitization to chronic cocaine and stress (Renthal et al., 2007). Mice lacking one allele of CBP [cAMP-response element binding protein (CREB) binding protein] have less accumulation of DeltaFosB as a result of decreased acetylation at the *FosB* promoter. This makes them less sensitive to chronic cocaine when compared to wild type mice having both alleles of CBP (Levine et al., 2005).

The transcription factor CREB can be induced by various stimuli including stress, hormones, growth factors and environmental stimuli. These stimuli modulate CREB by activation of the cAMP/PKA system. Phosphorylation at Ser-133 induces CREB activity (Shaywitz et al., 2000). CREB stimulates transcription by recruiting CBP. CREB has been implicated in neuronal plasticity and regulating long term memory consolidation (Guan et al., 2002). CREB functions in the dopaminergic limbic circuits in mammals to mediate responses to different drugs including opiates, cocaine, ethanol and morphine (Carlezon et al., 2005; McClung and Nestler, 2003; Nestler, 2004). Increased CREB

activity in the nucleus accumbens decreases sensitivity to morphine and cocaine and functions to form a feedback inhibitor pathway mediating tolerance to the effects of the drug (Carlezon et al., 1998).

CREB induced histone acetylation regulates *slowpoke* induced drug tolerance in flies

A specific spatio-temporal program of histone H4 acetylation across the *slowpoke* promoter mediates the up-regulation of the message following administration of the solvent anesthetic benzyl alcohol. This has been shown to be mediated by the Drosophila CREB gene, *dCREB2* (Wang et al., 2007).

The *slowpoke* promoter is a 7kb complex control region with 5 promoters and numerous control elements directing developmental and tissue-specific transcription. The ChromIP assay was used to detect histone H4 acetylation changes across the *slowpoke* promoter. The DNA associated with acetylated histone H4 was quantified by real-time PCR using primers specific for conserved regions of the promoter. Sequence conservation helped identify functionally important regions in the *slowpoke* transcriptional control region. Primers were picked to span conserved ~200 bp non-promoter fragments across the *slowpoke* promoter which included 4b, 6b, cre1, cre2, 55b. The neuronal and muscle promoter driven exons, C0, C1 and C2 were also assayed as was the C2C3 intronic region that has been shown to contain important control elements for *slowpoke* regulation (Figure 3.3; Benzyl Alcohol). A time course of histone acetylation revealed that 30 minutes post sedation there is no change across any of the regions assayed, but 4 hours after sedation there was a peak around 55b. By 6 hours, we observed a broad acetylation pattern in the vicinity of the neural promoters C0 and C1. At 24 hours this peak moved to a finely focused region over 6b, upstream of C1 and by 48 hours after BA sedation the acetylation levels were back to baseline (Figure 3.3; Benzyl Alcohol). Furthermore, these

acetylation changes were shown to require dCREB2 activity. Drug sedation was shown to activate the CREB signaling pathway. CREB was shown to occupy the promoter of *slowpoke* at conserved CRE binding sites to increase acetylation at conserved regions of the promoter. This causes message induction leading to behavioral tolerance (Wang et al., 2007).

I used the ChromIP assay to detect acetylation changes in the *slowpoke* promoter at 6, 24 and 48 hours following a single ethanol sedation. Since both benzyl alcohol and ethanol tolerance are dependent on transcriptional up-regulation of *slowpoke*, I expected to see increased acetylation of the promoter under the assumption that both of these drugs share a common regulatory theme at the promoter to cause message induction.

RESULTS

Ethanol sedation dynamically modifies the acetylation state of the *slowpoke* promoter.

ChromIP was performed on two groups of flies; ethanol treated and control. Chromatin was purified from fly heads that were collected 6, 24 and 48 hours after ethanol sedation. This material was sonicated to approximately 600 bp in length and immunoprecipitated using an antibody against anti-acetylated histone H4 (acH4). The choice of 600 bp was to ensure we covered the slowpoke transcriptional control regions and still had a small enough amplicon (~200 bp) to permit reliable quantification using the real-time PCR as longer amplification products are not optimal for real-time PCR. The choice of regions assayed span the whole length of the promoter in ~200 bp windows until the common coding exon (Figure 3.1A). The antibody recognizes H4 that has been acetylated at lysines K5, K8, K12 or K16 (*Upstate Biotechnology, NY, CAT#06-866*). The immunoprecipitate (IP) contains genomic DNA co-immunoprecipitated contained in

nucleosomes. Although genomic DNA from essentially the entire genome is present in the IP, the relative abundance of each portion of the genome is directly proportional to the acetylation state of the histones in the genome. By measuring the relative abundance of each part of the *slowpoke* transcriptional region in the IP, one obtains a measure of the relative acetylation state of chromatin in the original sample. The real-time PCR sybr green delta-delta CT method(ΔΔCT) was used to calculate the fold enrichment of acetylated histones in the ethanol versus control group. The *cyclophilin* promoter served as an internal control. The equation used to calculate fold change by the ΔΔCT method is: Fold change of histone modification $=2\text{^}(Ct^{input}\text{-}Ct^{IP})_{sedated}$ /$2\text{^}(Ct^{input}\text{-}Ct^{IP})_{control}$. The fold change of histone acetylation on the *cyclophilin* promoter was measured as a control and the value for each region assayed was normalized to the *cyclophilin* value.

The regions assayed were the non-promoter conserved elements 4b, 6b, cre1, scan2, 55b and the promoters C0 (neuronal), C1 (neuronal) and C2 (muscle). The intronic region C2C3 was also chosen as it contains putative control elements for *slowpoke* expression and has a CRE binding site (Figure 3.1A). Six hours after ethanol sedation increased acetylation levels were observed at 4b, 6b and 55b. This increase correlated with the message induction observed at 6 hours (Cowmeadow et al., 2006). By 24 hours the peak was shifted towards the neuronal promoter C0 and still elevated at 6b and cre1 (CREB binding motif near the neuronal promoter C1). By 48 hours after ethanol sedation, I observed a single significant acetylation peak over 6b. These results indicate that histone acetylation across the *slowpoke* transcriptional control region is dynamically modulated after ethanol sedation and after 48 hours we still observe a stable acetylation peak over a conserved region, 6b.

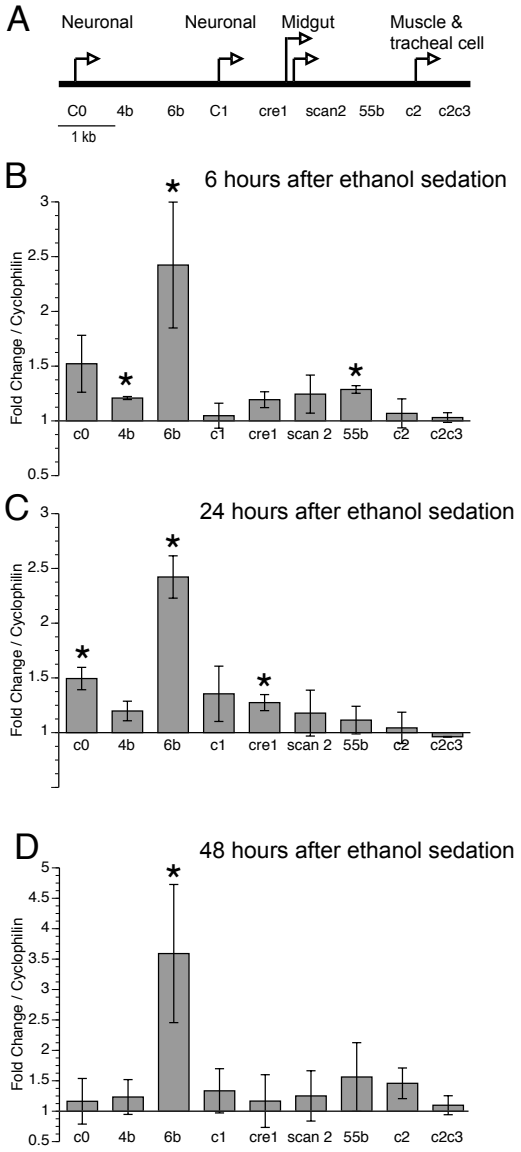


Figure 3.1. Ethanol sedation dynamically modifies the acetylation pattern of the *slo*wpoke control region. **A)** The *slowpoke* gene has five tissue-specific promoters. They are neuronal promoters C0 and C1; the midgut promoters C1b and C1c (indicated only by arrows); and the muscle/ tracheal cell promoter C2. These transcriptional start sites for the promoters are identified by the arrows. The conserved non promoter DNA motifs are represented below the line to delineate their 5' or 3' orientation respective to the promoters. **B-D)** Acetylation state of histone H4 surveyed after ethanol sedation as determined by chromatin immunoprecipitation and real-time PCR. Fold increase is the ratio of acetylation levels obtained for ethanol sedated animals and air treated age-matched controls. The fold

increase is normalized to the internal control, *cyclophilin*. **B)** Six hours after ethanol sedation there is a significant acetylation increase at 4b, 6b and 55b. **C)** At twenty-four hours later there is increased acetylation at C0 (neuronal promoter), 6b and cre1. **D)** By forty-eight hours after ethanol sedation the peak remains finely focused over 6b while returning to baseline levels in other regions. Bar graphs represent the mean ± SEM, and statistical analysis is by Student's t-test (n=3, *$p<0.05$).

DISCUSSION

Histone modifications mediate chromatin remodeling and this has been attributed to play a very important role in drug responses. Transcription factors implicated in drug responses such as CREB and DeltaFosB modulate the activity of multiple genes by controlling the acetylation state of the promoters (Nestler, 2004; Colvis et al., 2005). Cocaine administration in mice hyperacetylates the *Bdnf* and *cdk5* gene promoters mediated by the transcription factor DeltaFosB (McClung and Nestler, 2003; Colvis et al., 2005; Kumar et al., 2005). CREB mediated activation at *fosB* gene promoter promotes sensitivity to acute cocaine administration in mice (Levine et al., 2005). Histone deacetylases can also affect cocaine responses. Recently, it has been shown that chronic cocaine or stress decreases HDAC5 activity in the nucleus accumbens thereby promoting target gene activation by increased acetylation (Renthal et al., 2007). Thus, we see that in higher mammalian systems acetylation/deacetylation levels mediate responses to drugs of abuse.

The Drosophila *slowpoke* gene promoter has a very distinct spatio-temporal pattern of histone acetylation following benzyl alcohol sedation (Wang et al., 2007). The activation of CREB (dCREB2) is thought to mediate this dynamic response. The acetylation peaks are centered around conserved control elements in the promoter. At 4 hours following BA sedation we see a peak at 55b and by 6 hours a broad acetylation increase is evident across the the neuronal promoters C0, C1 and at 6b. By 24 hours, the peak has stabilized at 6b and returns to baseline levels by 48 hours (Figure 3.3; Benzyl Alcohol).

I have identified a similar spatio-temporal pattern of increased H4 acetylation at

important conserved elements in the *slowpoke* promoter following ethanol sedation. Histone acetylation changes are evident at 4b, 6b and 55b at 6 hours post ethanol administration. The magnitude of the peak at 55b is not as high when compared to the 6b peak after normalization to the internal control *cyclophilin*. By 24 hours, we see the increased acetylation shifting toward the neuronal promoter C0 and still remaining persistent at 6b. And by 48 hours, the peak remains stabilized at 6b while returning to baseline levels in other regions. s*lowpoke* message is up-regulated at 6 hours after sedation after which it rapidly declines and by 24 hours message abundance is back to baseline (From *Roshani Cowmeadow's Dissertation 2004*). This is correlated with behavioral tolerance seen at both 6 and 24 hours after the first sedative dose. The data from the ChromIP suggests that sedation produces a distinct acetylation peak around 6b in the *slowpoke* transcriptional control region that correlates with the observed message induction and behavioral tolerance. This is direct evidence that the induction of the gene is due to drug induced histone acetylation at the *slowpoke* promoter.

There are a few interesting observations when we compare the BA and ethanol ChromIP assays. Correlation of ChromIP data with message induction seems to differ among the two drugs. Following ethanol sedation we observe a persistent acetylation peak at a conserved DNA element (6b) 6, 24 and 48 hours after sedation. The peak at 6 hours correlates with the message induction observed at the same time-point after ethanol sedation (Cowmeadow et al., 2006). Though the peak is stable at 24 and 48 hours, the message returns to baseline levels by 24 hours (From *Roshani Cowmeadow's Dissertation 2004*). This contrasts with what we observed with benzyl alcohol sedation. After benzyl alcohol sedation message induction seems to coincide with the acetylation peak at 6b at 6 and 24 hours. By 48 hours, both message and acetylation levels return to

baseline (Wang et al., 2007).

So what could be the purpose of the persistent acetylation over 6b at 24 and 48 hours observed with ethanol? How does such a persistent acetylation lead to message down-regulation at 24 hours? One probability could be that acetylation at 6b promotes assembly of regulatory complexes in a temporal fashion so that by 24 hours a transcriptional repressor could be docked at 6b that down-regulates *slowpoke* expression beginning at 24 and lasting until 48 hours or even longer (we have not assayed later time-points). Using ChromIP we could assay for negatively acting transcription factors binding 6b and other conserved regions in the promoter. Figure 3.2 shows a sequence alignment of 55b and 6b from eight different Drosophila species. Many important neuronally expressed transcription factors have binding motifs in these regions. Some of these have inhibitory regulatory functions in the fly nervous system including CF-1 (Chorion Factor-1), En (Engrailed) and Dl (Dorsal). A functional ChromIP assay with antibodies against these transcription factors could reveal occupancy of an inhibitory transcription factor at 24 and 48 hours validating our hypothesis. It has also been observed that antagonistic histone modifications can occur on the same histone and regulate gene expression accordingly (Kouzarides, 2007). If such were the case, then we could assay for specific antagonistic modifications such as acetylation of lysines K14 and K18 (activation) and methylation at K9 and K27 (repression) in histone H3 as these are just a few residues apart on the same histone. We can also use antibodies against other known inhibitory PTMs in different histones and show occupancy at 6b at 24 and 48 hours. If we are able to demonstrate a repressive PTM in addition to the acetylation of histone H4 at 24 and 48 hours over 6b, then that would explain the message down regulation.

Another interesting difference between the benzyl alcohol and ethanol ChromIP is

that the acetylation peak at 6b seems to appear earlier with ethanol (6 hours) than with BA (24 hours) (Figure 3.3). The benzyl alcohol response starts off at 4 hours with increased acetylation at 55b. By 6 hours, the acetylation is centered around the neuronal promoters with only a smaller peak at 6b. This contrasts with the robust peak we see with ethanol over 6b at the same time-point (Figure 3.3). One reasonable explanation of these results could be that ethanol is a stronger inducer of the system than BA. Thus, we could see events unfolding at the promoter in a quicker time-scale with ethanol than with BA. An earlier ChromIP, preferably a time-course starting from 2-6 hours post ethanol sedation, would indicate if that were the case. If ethanol is indeed a stronger inducer of the system we should see an early 55b peak followed by a broad acetylation pattern over the neuronal promoters all happening within 6 hours of ethanol sedation. This would resemble the benzyl alcohol response albeit unfolding earlier at the promoter thus illustrating an important difference between the two drugs.

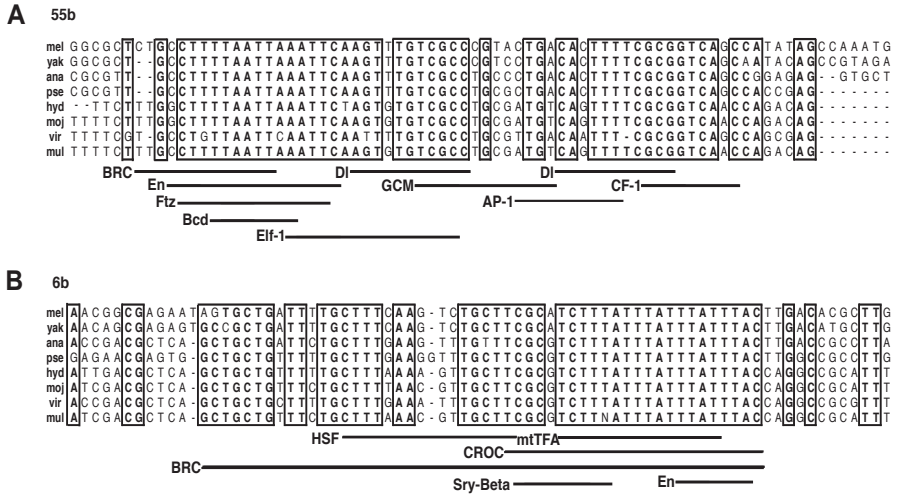


Figure 3.2. 55b and 6b genomic sequence is highly conserved across eight species of Drosophila.
This figure shows the sequence conservation of 55b and 6b across eight Drosophila species (From *Yan Wang's Dissertation 2007*).
A) Shows alignment of the 55b box and **B)** shows the alignment of 6b eight Drosophila species. The boxes around the sequences represent areas of highest identity (80%) and below the sequences the lines denote transcription factor binding motifs that are conserved in these species. Abbreviations: mel, *Drosophila melanogaster*; yak, *D. yakuba*; ana, *D. ananassae*; pse, *D. pseudoobscura*; moj, *D. mojavensis*, hyd, *D. hydei*; vir, *D. virilis*; mul, *D. mulleri*. Transcription factor binding sites: AP-1, Activator Protein-1; BRC, Broad Complex; Bcd, Bicoid; CF-1, Chorion Factor 1; CROC, Crocodile; DL, Dorsal; Elf-1, Element I-binding activity; En, Engrailed; Ftz, Fushi Tarazu; GCM, Glial Cells Missing; HSF, Heat Shock Motif; mtTFA, Mitochondrial Factor A; Sry-Beta, Serendipity Beta.

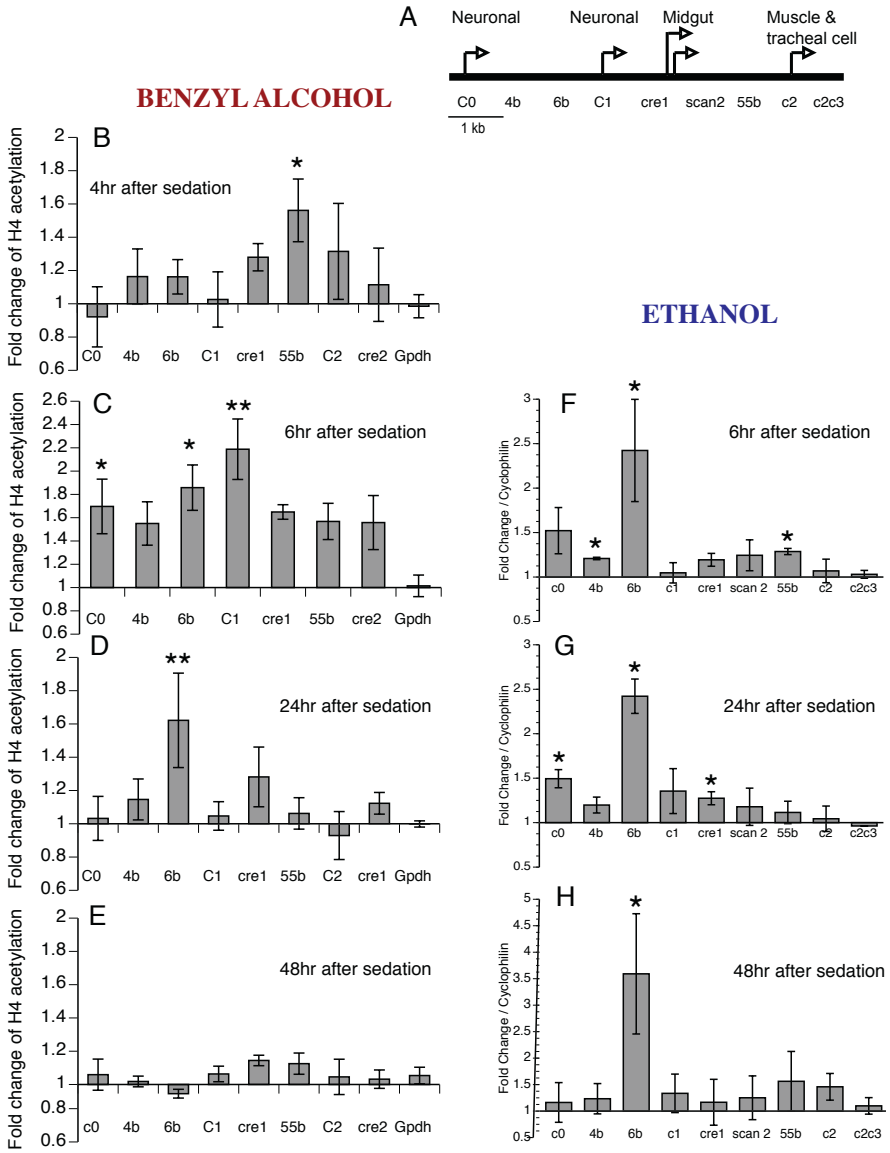


Figure 3.3. Comparison of the benzyl alcohol and ethanol acetylation patterns following a single sedative dose. **A)** The *slowpoke* gene promoter map showing five tissue-specific promoters. Neuronal promoters C0 and C1; the midgut promoters C1b and C1c (indicated only by arrows); and the muscle/tracheal cell promoter C2. These transcriptional start sites for the promoters are identified by the arrows. The conserved non promoter DNA motifs assayed by ChromIP are represented below the line to delineate their 5' or 3' orientation respective to the promoters. **B-E)** Acetylation state of histone H4 surveyed after benzyl alcohol (BA) sedation as determined by chromatin immunoprecipitation and real-time PCR. Fold increase is the ratio of acetylation levels obtained for BA sedated animals and mock-

treated age-matched controls. Bar graphs represent the mean ± SEM, statistical significance was determined by One-way ANOVA with Dunnett's post-hoc comparison (n=4-6, *p<0.05; **p<0.01 with respect to *Gpdh*). The values were not normalized to the internal control *Gpdh*, so the peak for the control is also shown. **B)** Four hours after benzyl alcohol sedation there was a significant increase in acetylation at conserved region 55b. **C)** Six hours after sedation, hyperacetylation was highest over promoter C1, although a second peak appeared to be centered over promoter C0. **D)** The acetylation state of histone H4 twenty-four hours after sedation had changed. Peak acetylation levels were centered at the conserved 6b region and the level of acetylation of other regions returned to control levels. **E)** By 48hrs acetylation level across the *slowpoke* promoter region returned to baseline levels (no change relative to the mock treated animals) (Modified from *Yan Wang's Dissertation 2007*).
F-H) Same as in **Figure 3.1 (B-D)**.
In the BA ChromIP scan2 was not assayed and in the ethanol ChromIP cre2 was not assayed.

Chapter 4: Tissue specific neuronal induction of the BK channel gene differentially regulates ethanol tolerance

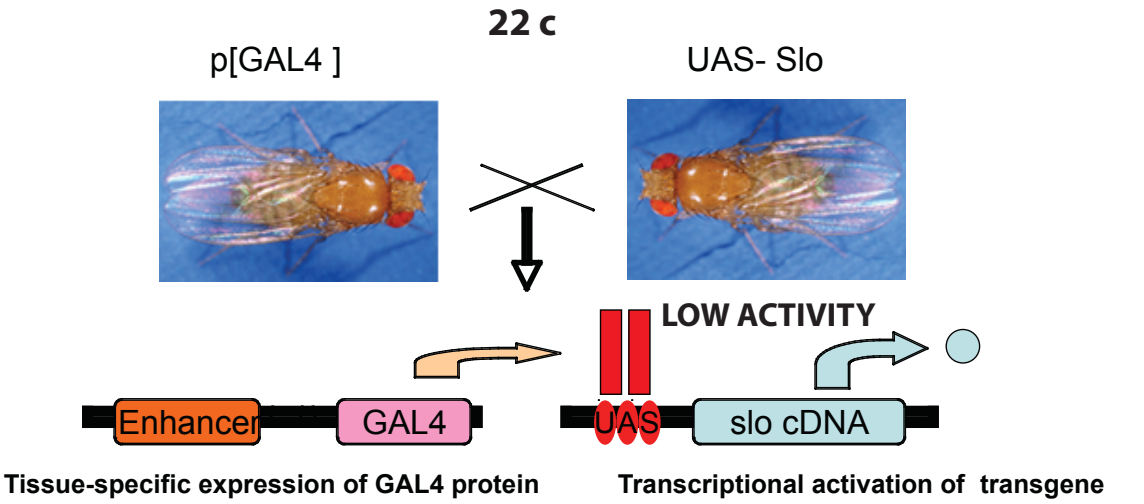
INTRODUCTION

Drug mediated molecular pathways are remarkably conserved among vertebrates and invertebrates. Drugs like cocaine are shown to affect conserved dopamine signaling pathways in flies and mammals (Nestler, 2005; Bainton et al., 2000). Circadian mutants have also been shown to affect cocaine sensitivity in flies and mice (Andretic et al., 1999; Liu et al., 2005). Ethanol affects the cAMP/PKA pathway in different model systems. In flies, defects in the cAMP pathway lead to ethanol sensitivity and one of the mutants characterized, *cheapdate* (*cpd*) is an allele of the memory mutant, *amnesiac*. These flies have defective cAMP signaling leading to ethanol sensitivity (Waddell et al., 2000; Moore et al., 1998). The cAMP pathway in mice mediates tolerance to ethanol sedation and the hypothermia associated with ethanol administration (Yang et al., 2003). Thus, we see that conserved neurotransmitter systems such as the dopaminergic system in flies are regulated by drugs such as cocaine and ethanol (Bainton et al., 2000). The mushroom bodies of the central brain in flies are the seat of olfactory learning and memory and regulate courtship conditioning, sleep and cAMP signaling (McGuire et al., 2001; Joiner et al., 2006; Waddell et al., 2000).

To characterize brain structures required for *slowpoke* mediated tolerance, the binary GAL4/UAS system was used to spatially target a *slowpoke* cDNA to specific brain regions (Brand and Perrimon, 1993). GAL4 is a yeast transcription factor which recognizes and binds the upstream activator sequence (UAS) (McGuire et al., 2004). GAL4 has an activation domain which recruits HATs and other transcription factors to stimulate transcription. The advantage of this system is that the components GAL4 and

UAS are bipartite existing in separate flies until we cross them. The fly community has a vast repertoire of GAL4 lines which express GAL4 in a spatio-temporal fashion in the nervous system of the fly. To conditionally express *slowpoke* in different parts of the brain, we built a UAS-Slo construct. It was built by subcloning the *slowpoke* cDNA contained in the B52 transgene into the pUAST vector downstream of the UAS sequence.

The crosses were set up with p[GAL4] lines expressing *slowpoke* in the learning and memory centers of the Drosophila brain, the mushroom bodies (MB) (Rodan et al., 2002). Additional GAL4 lines targeting neurotransmitter systems such as dopaminergic and cholinergic neurons were chosen because drugs such as cocaine and ethanol are shown to regulate dopaminergic pathways in the nucleus accumbens and striatum in higher vertebrates (Nestler, 2005).



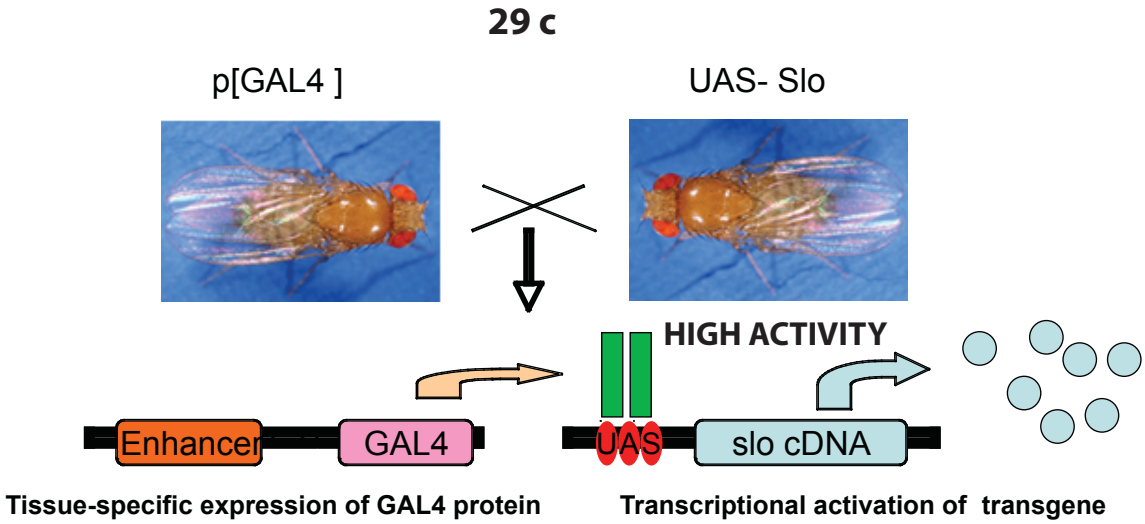


Figure 4.1. Using the temperature dependence of the GAL4/UAS conditional expression system to induce *slowpoke*. GAL4 protein activity is temperature dependent. We used 29 ºC to maximally induce the UAS-Slo transgene compared to 22 ºC (Figure 4.3). Induction of *slowpoke* in a tissue specific pattern could be controlled depending on the p[GAL4] enhancer expression pattern.

RESULTS AND DISCUSSION

Three p[GAL4] lines were used to drive a *slowpoke* cDNA in the adult fly brain. The p[GAL4] line 854-OK107 drives GAL4 expression in the mushroom bodies of the fly. The lines 6798 and 7010 preferentially drive expression in the cholinergic and dopaminergic/serotonergic neurons in the fly respectively.

One of the problems we anticipated when assaying for ethanol resistance was genetic background effects when comparing the offspring to the parental lines. To minimize this variability, I used the property of GAL4 wherein the protein's activity can be modulated by heat incubation (Duffy, 2002; Jarrett, 2000) (Figure 4.1). The protein activity is minimal at 16 ºC and optimal at 29 ºC. The flies were not incubated at temperatures higher than 30 ºC to minimize any adverse phenotypical effects. Crosses were setup and the age-matched female progeny divided into two groups, one group was incubated at 22 ºC and the other at 29 ºC. Both the incubations were done in humidified chambers for 3 days (I chose 22 ºC as the lower temperature instead of 16 ºC to avoid long generation times and differences in eclosion of progeny). All crosses were maintained at 22 ºC and after eclosion, I moved half of the progeny to 29 ºC for 3 days.

One of the first things I had to address was the prolonged heat incubation. Although wild type flies can tolerate 29 ºC and show no morbidity or mortality, I wanted to assess the effects of such prolonged heat shock on ethanol tolerance. In Chapter 2, our heat shock control experiments for B52H resistance showed no effect due to heat. However, the heat shock protocol for B52H was a brief 30 minute heat shock at 37 ºC (Figure 2.1C-D). The controls for *shi*^{ts2} and *para*^{ts1} experiments underwent a 5 hour heat shock at 30 ºC and still showed no effect due to heat (Chapter 5) (Figure 5.5B). Since

none of the above heat incubations were longer than a day, I performed a control experiment for the 3 day 29 ºC protocol.

Wild type, Canton S(CS) flies show ethanol resistance with the heat induction protocol.

I incubated wild type, Canton S (CS) flies at 25 ºC (control) and 29 ºC (experimental) for three days. On the third day, they were subjected to the tolerance assay. In the tolerance assay, both groups received a saturated ethanol stream until they were completely sedated. The ethanol stream was then switched to humidified air and return of postural control was assayed every minute. I observed that flies incubated at 29 ºC acquired resistance to ethanol sedation (Figure 4.2A). When I performed this experiment, I used 25 ºC instead of 22 ºC for the control group . Nevertheless, I observed a difference in recovery despite the fact that there was only a 4 ºC difference between the two groups. All future control heat shock paradigms were done in an incubator specifically maintained at 22 ºC. The above heat shock paradigm made flies resistant to ethanol sedation. We believe this is pharmacokinetic tolerance due to higher temperatures causing increased metabolic activity in flies thus leading to rapid breakdown of ethanol and causing tolerance.

Pharmacokinetic tolerance due to heat is not dependent on slowpoke.

To determine whether the tolerance due to heat was *slowpoke* independent, I tested flies mutant for the *slowpoke* gene (slo^4). I subjected slo^4 flies to a 1 day heat incubation at 29 ºC instead of 3 days because these mutants are not very healthy and a 3 day incubation at elevated temperatures would cause significant mortality. slo^4 flies were divided into 2 groups and one group incubated at 29 ºC (HS) for 1 day and the control group at 22 ºC (Control) for 1 day. At the end of 24 hours, they were immediately tested

for ethanol tolerance and recovery from sedation assayed. I observed that *slo*^{4} flies exhibited heat induced ethanol resistance (Figure 4.2B).

I also measured *slowpoke* message from the neuronal exon C1 in wild type flies (CS) incubated at 29 ºC for three days and we did not see an up-regulation of *slowpoke* relative to *cyclophilin*. Therefore, the tolerance seen in these flies is not due to a transcriptional up-regulation of *slowpoke*. These results seem to indicate the observed tolerance is *slowpoke* independent and is probably pharmacokinetic in nature.

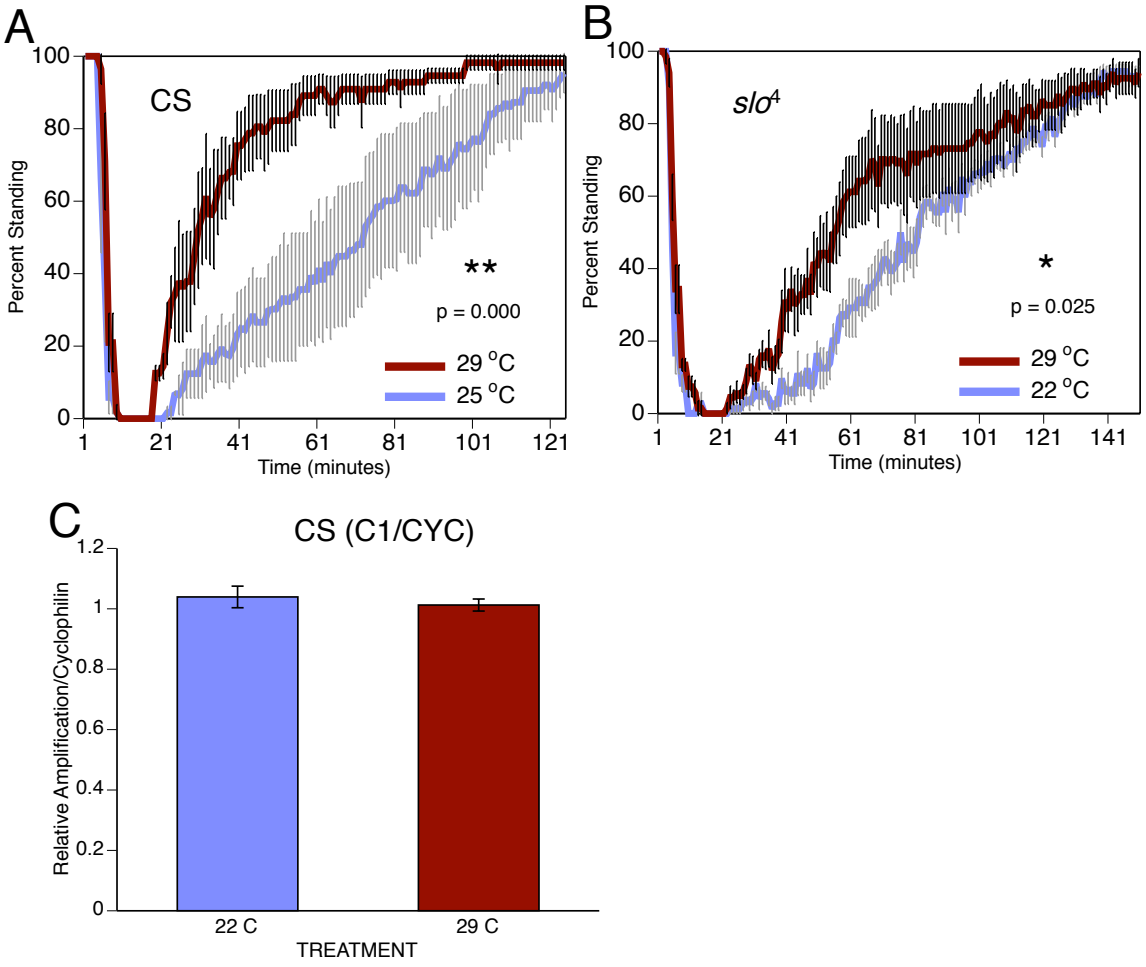


Figure 4.2. Heat incubation at 29 ºC causes ethanol resistance that is not dependent on *slowpoke*. **A)** CS flies were age matched and divided into two groups. One group was incubated at 25 ºC for 4 days and the other group incubated at 29 ºC for 4 days. On the fourth day a tolerance assay was performed where both groups received ethanol. Recovery was assayed after switching the ethanol stream to air and counting the number of flies which regained postural control. We see that a prolonged heat incubation of wild type flies causes ethanol resistance. **B)** *slo*^{4} flies were age matched and divided into two groups. One group was incubated at 22 ºC for 1 day and the other group incubated at 29 ºC for 1 day. On the second day a tolerance assay was performed where both groups received ethanol. Recovery assayed after switching the ethanol stream to air and counting the number of flies which regained postural control. We observe that *slo*^{4} can become resistant to ethanol sedation after one day at the higher temperature. Statistical analysis is by the log rank test. This accounts for the differences in recovery of the whole population between the two

curves (*p<0.05; **p<0.01). The p-values are indicated on the graphs if significant. Error bars are SEM for each data point. **C)** Total RNA made from CS flies was used in a reverse transcription reaction with oligo dT primers (Invitrogen®) and assayed by real time PCR for C1 mRNA abundance relative to *cyclophilin*. The two treatment groups were 22 ºC and 29 ºC for 3 days. We observe that heat incubation does not induce the *slowpoke* message. The error bars are SEM. n=3.

In order to establish that this is a pharmacokinetic tolerance, we would have to measure ethanol breakdown using a gas chromatograph assay (Cowmeadow et al., 2005). The absolute amount of ethanol remaining in the body of the fly could be measured at specific time points during the recovery phase. Comparison of the breakdown slopes of both the control and experimental flies would indicate the efficacy of breakdown in both groups. If the breakdown is faster in the flies incubated at 29 ºC group versus flies incubated at 22 ºC it indicates that the tolerance seen in CS and *slo*^{4} flies is a pharmacokinetic response. Behaviorally this could be done by incubating flies at 22 ºC immediately following a 29 ºC incubation and then testing for ethanol resistance. If the resistance phenotype is not evident after flies have had a chance to equilibrate at 22 ºC, then it indicates that the observed tolerance is pharmacokinetic and arises due to the higher metabolic activity at 29 ºC.

Modified heat induction protocol.

The heat shock protocol was modified to address whether the tolerance I saw with the controls was indeed pharmacokinetic in origin. I used a modified heat shock protocol in which flies were incubated at 29 ºC for 3 days and shifted to 22 ºC for 1 day. This allows time for the increased metabolic activities to come back to baseline. On the fourth day, these flies were compared to the uninduced group incubated at 22 ºC for 4 days. The tolerance assay was done on the fourth day and recovery from sedation assayed. The control wild-type flies (CS) did not acquire ethanol resistance due to heat with the updated protocol (Figure 4.4A). This indicated that the earlier observed tolerance was pharmacokinetic in origin resulting from increased metabolic activity. The modified heat induction protocol was used for all further behavioral experiments with the GAL4/UAS progeny.

Temperature dependent GAL4 activation induces UAS-Slo.

In order to ascertain the efficacy of GAL4 temperature activity, I measured the transgenic induction of *slowpoke* after temperature induction. To test whether the increased activity of GAL4 causes up-regulation of *slowpoke* cDNA in the induced versus uninduced progeny, I used multiple GAL4 lines and set up crosses with the UAS-Slo line.. The p[GAL4] lines used were a pan-neuronal driver (*nrv2*), two mushroom body drivers (854 and 6906), a cholinergic system driver (6798) and a dopa/5HT driver (7010). Crosses were set up and the progeny were collected and incubated at the two temperatures, 22 ºC (uninduced) and 29 ºC (induced) for 3 days. On the third day, total RNA was extracted and the message measured by quantitative real-time PCR with primers specific to the *slowpoke* cDNA sequence in the transgene. Message abundance was normalized to *cyclophilin*. As expected, the temperature dependent GAL4 activity consistently up-regulated the UAS-Slo encoded *slowpoke* cDNA consistently in all the crosses (Figure 4.3).

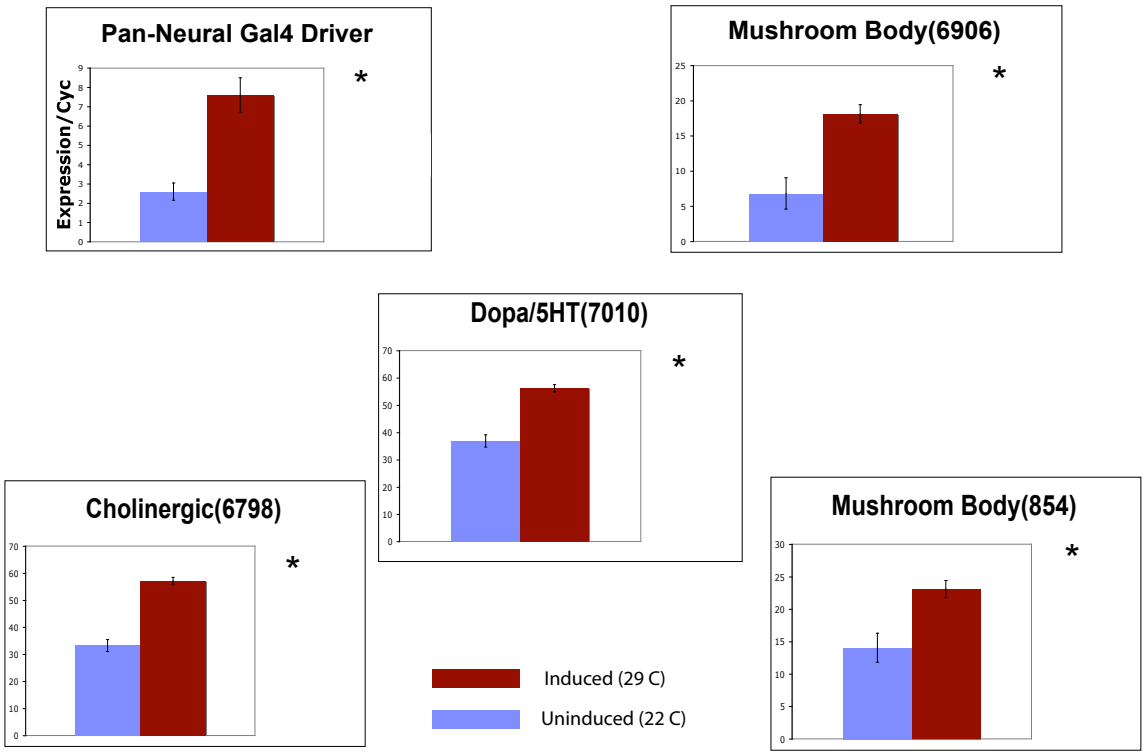


Figure 4.3. Enhanced GAL4 activity at higher temperature induces *slowpoke* message from the transgene consistently in multiple lines. Total RNA was made from the progeny of the GAL4 X UAS-Slo crosses. The different drivers used are indicated on the individual figures. The pan-neuronal driver used was *nrv2*. The progeny from the crosses were collected and split into two groups that were incubated at the two temperatures 29 ºC (induced) for the experimental group and 22 ºC (uninduced) for the control group. After 3 days RNA extracted and used in a reverse transcription reaction with gene specific primers for the UAS-Slo transgene and *cyclophilin*. Real time PCR was used to quantify the message abundance normalized to *cyclophilin*. We observed that the induction protocol resulted in transgenic up-regulation of the *slowpoke* cDNA consistently among all the lines tested. The error bars are SEM. n=3 *p<0.05 Student's t-test.

Tissue specific *slowpoke* induction has pleiotropic effects on ethanol tolerance.

Crosses were set up with three p[GAL4] lines; the mushroom body expressing GAL4 (854-OK107); GAL4 expressed in the dopaminergic/serotonergic neurons (7010) and GAL4 expressed in the cholinergic neurons of the brain (6798).

UAS-Slo induction in the mushroom bodies is required for slowpoke dependent tolerance.

The mushroom body p[GAL4] line 854-OK107 was crossed to the UAS-Slo line and the progeny tested for ethanol resistance. Age matched female flies were divided into two groups; induced (29 ºC) and uninduced (22 ºC). They were treated according to the modified heat induction protocol to minimize the effects of heat induced pharmacokinetic tolerance. The tolerance assay done on the fourth day revealed that inducing *slowpoke* in the mushroom bodies phenocopied resistance, implicating a role for these structures in *slowpoke* mediated tolerance (Figure 4.4B).

Mushroom bodies are critical structures in the fly brain involved in olfactory learning and classical conditioning (McGuire et al., 2001; Pascual and Preat, 2001). They play important regulatory roles in sleep that is modulated via the cAMP/PKA pathway (Joiner et al., 2006). Olfactory learning mutants such as *amnesiac*, *rutabaga*, and the cell adhesion molecule *fasciclin*II show altered ethanol sensitivity implicating the cAMP pathway as an important modulator of drug responses in flies (Moore et al., 1998; Cheng et al., 2001). We have shown that CREB occupancy at conserved CRE sites in the *slowpoke* promoter increases after benzyl alcohol sedation and activation of a CREB dominant negative transgene inhibits *slowpoke* message induction (Wang et al., 2007). The cAMP signaling pathway is responsible for drug effects in higher eukaryotes. In

mice, the cAMP pathway regulates ethanol sensitivity (Pandey et al., 2005; Thiele et al., 2000). These observations bolster our results suggesting that the mushroom bodies in the fruit fly are an important neuroanatomic loci for *slowpoke* mediated ethanol tolerance.

UAS-Slo induction in the Dopa/5HT neurons causes sensitization to ethanol sedation.

The p[GAL4] line 7010 carries an enhancer driving GAL4 in the dopaminergic/ serotonergic neurons. The flies were crossed to the UAS-Slo line and the progeny tested for ethanol resistance. Age matched female flies were split into two groups, induced (29 ºC) and uninduced (22 ºC) and incubated according to the modified heat induction protocol. The tolerance assay done on the fourth day revealed that inducing *slowpoke* in the dopaminergic/serotonergic neurons causes sensitization to ethanol sedation (Figure 4.4C). This is not due to differences in transgenic message induction as we observed an up-regulation of *slowpoke* message compared to the uninduced flies (Figure 4.3).

The GAL4 driver in 7010 (ddc-GAL4) expresses *slowpoke* in the dopaminergic neurons in the fly. In mammalian systems, we see that drugs such as cocaine and amphetamine act to increase sensitization of dopaminergic pathways. This can lead to acute reinforcement of reward pathways leading to addiction (Nestler, 2005). In flies, depletion of dopamine levels, by feeding flies 3iodotyrosine (3IY), reduces behavioral responses to cocaine and nicotine. The behavioral hyperactivity induced by cocaine and nicotine is reduced and flies exhibit decreased sensitivity to the drugs. (Bainton et al., 2000). Ethanol has been shown to affect dopaminergic signaling. Acute ethanol administration increases locomotor activity in rats via dopamine release (Di Chiara and Imperato, 1986). In flies, ethanol induced hyper-activity is reduced by dopamine depletion. This effect is reversible by administering L-Dopa to flies (Bainton et al., 2000). Expressing *slowpoke* in the dopaminergic neurons gives rise to ethanol sensitization in

our assay. We hypothesize that increased *slowpoke* augments the pre-synaptic release of dopamine that then causes increased sensitization to an acute dose of ethanol. If this were true, then inhibiting dopamine synthesis pharmacologically (3IY) or ablating the dopaminergic neurons by using UAS-toxins should reduce the sensitivity of flies to ethanol.

UAS-Slo induction in the cholinergic neurons shows no effect on ethanol tolerance.

The p[GAL4] line 6798 carries an enhancer driving GAL4 in the acetyl cholinergic neurons in the fly brain. The flies were crossed to the UAS-Slo line and the progeny tested by the modified heat induction protocol. The tolerance assay revealed that inducing *slowpoke* in the cholinergic neurons caused no significant change from uninduced flies (Figure 4.4D).

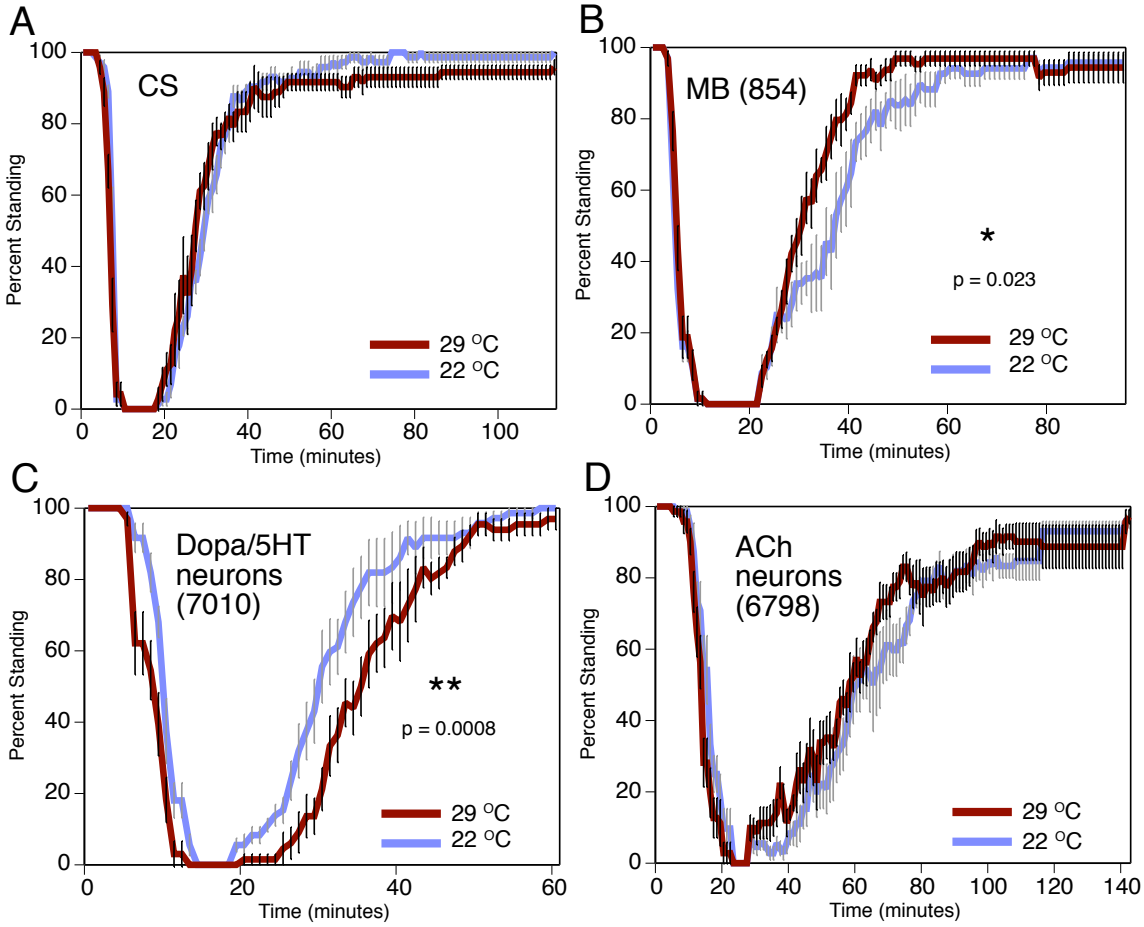


Figure 4.4. Induction of *slowpoke* in the mushroom bodies causes resistance and in the dopa/5HT neurons causes sensitization. **A)** CS flies were age matched and divided into two groups. One group was incubated at 22 ºC for 4 days [22 ºC] and the other group incubated at 29 ºC for 3 days and 22 ºC for 1 day [29 ºC]. This was the modified heat induction protocol. On the fourth day a tolerance assay was performed where both groups received ethanol. Recovery assayed after switching the ethanol stream to air and counting the number of flies which regained postural control. We observed that heat induction with this modified protocol does not cause pharmacokinetic tolerance and so this protocol was used for the GAL4/UAS-Slo experiments. **B-D)** The males from the p[GAL4] lines 854 (MB), 7010 (Dopa/5HT) and 6798 (ACh) were crossed to UAS-Slo females and the progeny collected and split into two groups. The heat incubation was done as detailed above for the CS (modified heat induction protocol). On the fourth day a tolerance assay was performed where both groups received ethanol. Recovery assayed after switching the ethanol stream to air and counting the number of flies which regained postural

control. **B)** The mushroom body (854) inducing UAS-Slo (29 ºC) acquires ethanol resistance when compared to the reduced *slowpoke*-inducing controls (22 ºC). **C)** In the Dopa/5HT (7010) expressing line we see sensitization with *slowpoke* induction (29 ºC) when compared to controls (22 ºC). **D)** *Slowpoke* induction (29 ºC) in the acetyl cholinergic neurons (6798) does not cause any significant change from controls (22 ºC).
Statistical analysis is by the log rank test. This accounts for the differences in recovery of the whole population between the two curves (*$p<0.05$; **$p<0.01$). The p-values are indicated on the graphs if significant. Error bars are SEM for each data point.

The mechanisms of alcohol action are only partially understood and require detailed characterization of the brain regions involved in mediating the different phenotypes such as sensitization, tolerance, dependence, withdrawal and addiction. Characterizing the neuroanatomic loci critical for *slowpoke* mediated ethanol tolerance in Drosophila would be very useful in identifying potential epistatic candidate genes involved in ethanol tolerance. The GAL4/UAS experiments implicate *slowpoke* as a critical mediator of rapid tolerance in the mushroom bodies of the fly brain, whereas in the dopa/5HT neurons, *slowpoke* acts to cause sensitization to ethanol. The induction from the transgene (GAL4/UAS-Slo) is up-regulated in both the lines and cannot be attributed to the difference in behavioral outcomes observed. Such a pleiotropic response warrants further characterization using genetic tools. Mutations affecting mushroom body development [*mushroom body miniature* (*mbm*); *mushroom body defective* (*mbd*)] can be assayed for ethanol tolerance. Targeting toxins to specific neurotransmitter systems and manipulating dopaminergic transmission pharmacologically would tell us whether these pathways have conserved ethanol responses in flies and help point us to similar molecular pathways and analogous structures in higher eukaryotes that are involved in ethanol tolerance.

Chapter 5: Tolerance is a cell autonomous property of the nervous system

INTRODUCTION

A cell autonomous property of a system is that quality mediated by defined local circuits or pathways that make up the system. In other words, it is independent of cellular communication. An emergent property is one where the outcome of an event cannot be attributed functionally to a specific region. It involves communication between different regions to bring out the desired phenotype.

Ethanol tolerance in flies is a neuronal phenotype. Following a brief ethanol sedation, the molecular pathways responsible for tolerance are initiated probably at specific regions in the brain that involve changes in gene expression at multiple loci resulting in regulation of neuronal communication. In order to test whether this tolerance requires cellular communication at the synaptic level, we blocked neuronal signaling in the brain and assayed for tolerance. If blocking neuronal signaling also blocked the acquisition of tolerance, it would indicate that tolerance is as an emergent property of the nervous system as opposed to being a cell autonomous one.

Drosophila has a number of well characterized temperature sensitive mutants that exhibit paralysis at restrictive temperatures and recover when moved to permissive temperatures. I used three neuronal temperature sensitive mutants *shi*^{ts2}, *para*^{ts1} and *comt*^{tp7} for blocking synaptic transmission in the nervous system.

The *shibire* gene encodes for the Drosophila homolog of the synaptic vesicle recycling protein, DYNAMIN. The allele *shi*^{ts2} is a temperature sensitive allele that exhibits reversible paralysis at the restrictive temperature (30 ºC) (Grigliatti et al., 1973;

Koenig et al., 1983). This is due to a conditional blockade of synaptic transmission due to defective vesicle recycling.

The allele *para*^{ts1} (*paralytic*) is a mutation in the α-subunit of the voltage gated sodium channel gene in the fly. It is a temperature sensitive allele which blocks action potential propagation and subsequent evoked vesicle fusion at the restrictive temperature of 30 ºC (Loughney et al., 1989).

The allele *comt*^{tp7} (*comatose*) is a temperature sensitive mutant allele of the Drosophila NSF protein (dNSF1). It is critical for vesicle fusion at the synapse and exocytosis. The mutant phenotype is manifested at the restrictive temperature (35 ºC) (Siddiqi and Benzer, 1976). At restrictive temperatures, *comt*^{tp7} flies have buildup of 7S synaptic SNARE complexes and so the recovery from paralysis is not as instantaneous as observed with *shi*^{ts2} and *para*^{ts1} mutants.

The synaptic blockade was initiated by a heat shock immediately following the first ethanol sedation on day 1. On day 2 the flies were tested for ethanol tolerance. On day 1, I used a 5 hour heat shock at 30 ºC for both *shi*^{ts2} and *para*^{ts1} mutants. For the *comt*^{tp7} flies, the heat shock paradigm was modified to 35 ºC for 30 minutes. A shorter duration of heat shock was used with *comt*^{tp7} flies because the paralysis due to the allele does not recover instantaneously upon reverting back to permissive temperatures. This contrasts with the rapid recovery observed in both *shi*^{ts2} and *para*^{ts1} mutants (Siddiqi and Benzer, 1976). The delay in recovery in *comatose* mutants has been attributed to the time taken for dissociation of the 7S complexes that have built up during the exocytosis block (Littleton et al., 1998). Nevertheless, these flies do not have any associated mortality or phenotypic defects over the course of their recovery and are comparable to their sibling

non-heat shocked controls.

RESULTS

The three mutant lines (*shi*^{ts2}, *para*^{ts1} and *comt*^{tp7}) were age matched and subjected to the following experimental protocols.

The control experiments for each mutant were performed first. The first control I performed was to see if the flies could acquire ethanol tolerance at the permissive temperature. The flies were divided into two groups that were exposed to fresh air (Control) versus ethanol saturated air (Ethanol) on day 1. On day 2, both groups were subjected to the tolerance assay and recovery from sedation assayed by return of postural control (**A**).

The second control was the effect of the heat alone (HS) on ethanol tolerance. The flies were treated accordingly 30 ºC for 5 hours (*shi*^{ts2} and *para*^{ts1}) and 35 ºC for 30 minutes (*comt*^{tp7}). They were compared to flies that were exposed to fresh air (Control) on day 1. On day 2 both groups were subjected to the tolerance assay and recovery from sedation assayed by return of postural control (**B**).

The third experiment was the heat shock following ethanol sedation on day 1. Flies which received ethanol immediately followed by a heat shock (30 ºC 5 hours for *shi*^{ts2} and *para*^{ts1}; 35 ºC 30 minutes for *comt*^{tp7}) were compared to flies which received heat shock alone (HS) on day1. On day 2 both groups received ethanol and assayed for tolerance **(C**).

I chose a 5 hour heat shock to ensure we blocked neuronal transmission long enough to interfere with the acquisition of tolerance. If the flies were not able to acquire tolerance on day 2, then it would indicate that neuronal communication is required for

tolerance and that it is an emergent property of the nervous system. If the flies were still able to acquire tolerance that would indicate that tolerance is a cell-autonomous property of the brain.

The above three experiments (A, B and C) were also done on wild type, Canton S (CS) flies to observe the effects of heat shock on ethanol tolerance in wild type flies (Figure 5.5).

Synaptic blockade due to *shi*^{ts2} can block the acquisition of tolerance.

The first control experiment performed was to test *shi*^{ts2} flies for normal ethanol tolerance (Figure 5.1A). For this experiment I took age matched female *shi*^{ts2} flies and divided them into control (Control) and ethanol (Ethanol) groups which received air and a saturated ethanol stream respectively on day 1. On day 2 both groups were subjected to the tolerance assay. The *shi*^{ts2} flies were able to acquire ethanol tolerance (Figure 5.1A).

The second control experiment was performed to assess the effects of a 30 ºC heat shock for 5 hours on ethanol tolerance in *shi*^{ts2} flies (Figure 5.1B). In this experiment, the control group (Control) received air and the heat shock group (HS) was subjected to a 30 ºC heat shock for 5 hours in a humidified chamber. After 5 hours both groups were moved to food vials. On day 2 I performed a tolerance assay. There was no significant difference in the recovery curve for both groups, so heat by itself did not cause *shi*^{ts2} flies to acquire ethanol tolerance (Figure 5.1B).

Following the controls the third experiment was done with two groups of flies treated as follows. The first group of flies received a 30 ºC heat shock for 5 hours (HS) on day 1. The experimental group received ethanol sedation and as soon as all the flies were sedated, were immediately shifted to a 30 ºC humidified chamber for 5 hours

(Ethanol+HS) (Figure 5.1C). On day 2, the flies were subjected to the tolerance assay and recovery assayed every minute by looking for restoration of the righting reflex. In figure 5.1C, we observe that the flies do not acquire tolerance. Inhibiting neurotransmission blocks the acquisition of tolerance in *shi*^{ts2} flies.

The *shibire* experiments show that we can block the acquisition of tolerance by inhibiting synaptic communication in the brain and suggest that tolerance is an emergent property of the nervous system.

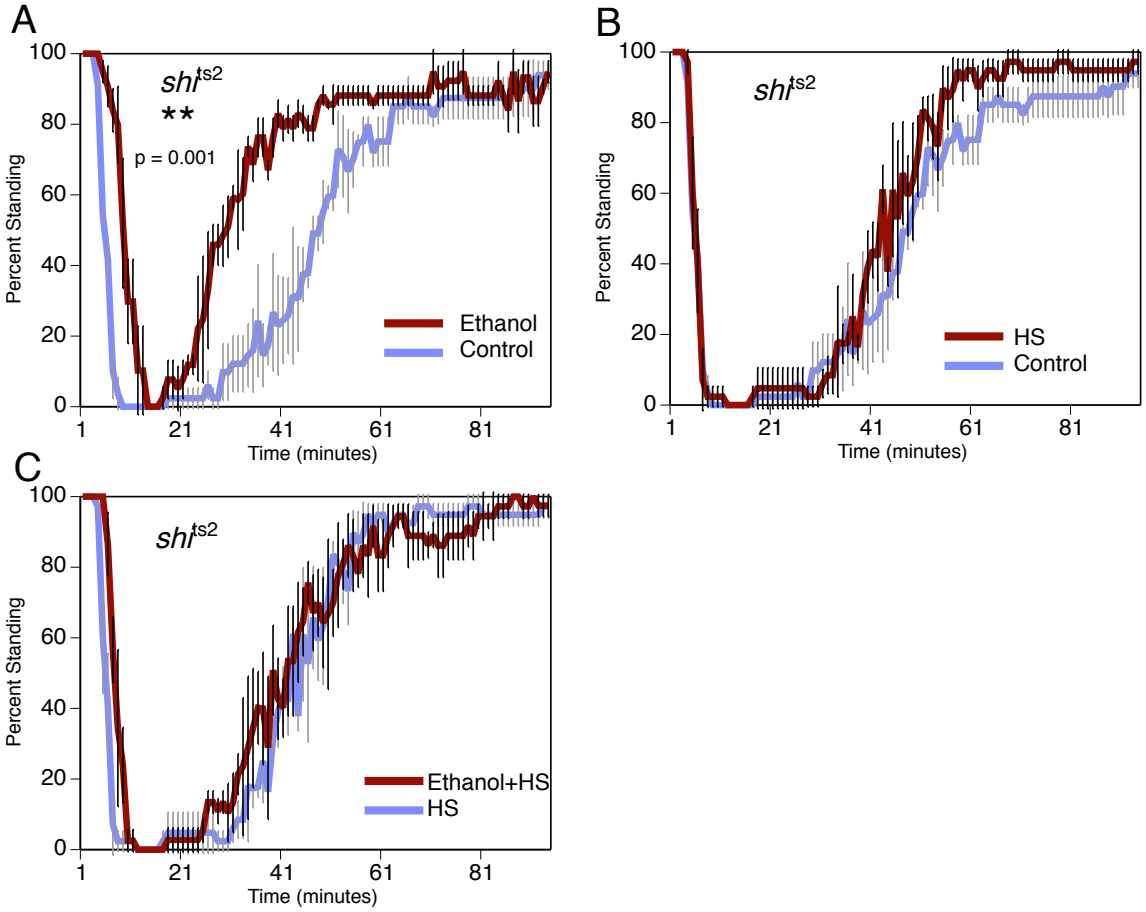


Figure 5.1. Acquisition of tolerance is blocked by *shi*^{ts2}. **A)** *shi*^{ts2} flies were age matched and divided into two groups. Control flies received air (Control) and the ethanol group received an ethanol sedation (Ethanol) on day 1. After 24 hours a tolerance assay was performed where both groups received ethanol till they were sedated. Recovery assayed after switching the ethanol stream to air and counting the number of flies which regained postural control. We observed *shi*^{ts2} flies acquire ethanol tolerance at the permissive temperature. **B**) *shi*^{ts2} flies were age matched and divided into two groups. Control flies received air (Control) and the second group received a 30 ºC heat shock for 5 hours (HS) on day 1. After 24 hours a tolerance assay was performed where both groups received ethanol till they were sedated. Recovery assayed after switching the ethanol stream to air and counting the number of flies which regained postural control. We observed that heat shock alone in *shi*^{ts2} flies does not cause ethanol resistance. Heat shock can substitute for control. **C**) *shi*^{ts2} flies were age matched and divided into two groups. Heat shock group received a 30 ºC heat shock for 5 hours (HS) on day 1. The second group received an

ethanol sedation immediately followed by a heat shock incubation at 30 ºC for 5 hours (Ethanol+HS). After 24 hours a tolerance assay was performed where both groups received ethanol till they were sedated. Recovery assayed after switching the ethanol stream to air and counting the number of flies which regained postural control. We observed that heat shock can block the acquisition of tolerance in *shi*^{ts2} flies.
Statistical analysis is by the log rank test. This accounts for the differences in recovery of the whole population between the two curves (*p<0.05; **p<0.01). The p-values are indicated on the graphs if significant. Error bars are SEM for each data point.

Synaptic blockade due to *para*^{ts1} does not block the acquisition of tolerance.

The first control experiment performed was to test *para*^{ts1} flies for normal ethanol tolerance (Figure 5.2A). For this experiment, I took age matched female *para*^{ts1} flies and divided them into control (Control) and ethanol (Ethanol) groups that were exposed to fresh air and a saturated ethanol stream respectively on day 1. On day 2 both groups were subjected to the tolerance assay. The *para*^{ts1} flies were able to acquire ethanol tolerance at the permissive temperature (Figure 5.2A).

The second control experiment was performed to assess the effects of a 30 ºC heat shock for 5 hours on ethanol tolerance in *para*^{ts1} flies (Figure 5.2B). At elevated temperatures there is a reduction in action potential generation and propagation in *para*^{ts1} flies due to reduced sodium channels in the neuron (Nelson and Wyman, 1990). I wanted to assess the effect of the heat induced neuronal paralysis alone on ethanol resistance. In this experiment, the control group (Control) received air and the heat shock group (HS) was subjected to a 30 ºC heat shock for 5 hours in a humidified chamber. After 5 hours, both groups were moved to food vials. On day 2, I performed a tolerance assay and observed there was no significant difference in the recovery curve for both groups. Heat shock by itself did not cause *para*^{ts1} flies to acquire ethanol tolerance (Figure 5.2B).

Following the controls, the third experiment was done with two groups of flies treated as follows. The first group of flies received a 30 ºC heat shock for 5 hours (HS) on day 1. The experimental group received ethanol sedation and as soon as all the flies sedated, they were immediately shifted to a 30 ºC humidified chamber for 5 hours (Ethanol+HS) (Figure 5.2C). On day 2, the flies were subjected to the tolerance assay and recovery assayed every minute by looking for restoration of the righting reflex. In figure

5.2C, we observe that the flies are able to acquire ethanol tolerance. Inhibiting neurotransmission does not block the acquisition of tolerance in *para*^{ts1} flies. This indicates that tolerance is a cell autonomous property of the nervous system, but this result contradicted what we saw with *shi*^{ts2} flies (Figure 5.1).

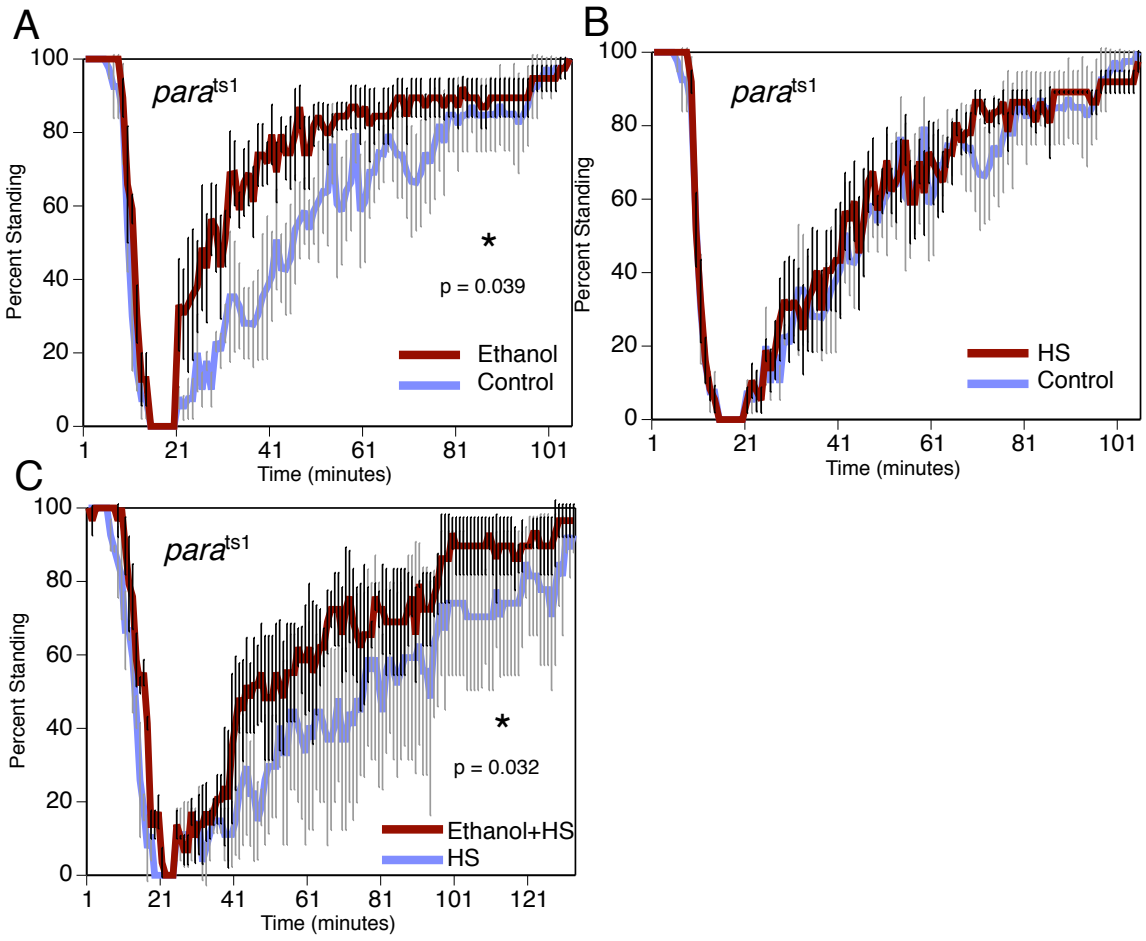


Figure 5.2. Acquisition of tolerance is not blocked by *para*^{ts1}. **A)** *para*^{ts1} flies were age matched and divided into two groups. Control flies received air (Control) and the ethanol group received an ethanol sedation (Ethanol) on day 1. After 24 hours a tolerance assay was performed where both groups received ethanol till they were sedated. Recovery assayed after switching the ethanol stream to air and counting the number of flies which regained exhibited the righting reflex. We observed *para*^{ts1} flies acquire ethanol tolerance at the permissive temperature. **B**) *para*^{ts1} flies were age matched and divided into two groups. Control flies received air (Control) and the second group received a 30 ^{o}C heat shock for 5 hours (HS) on day 1. After 24 hours a tolerance assay was performed where both groups received ethanol till they were sedated. Recovery assayed after switching the ethanol stream to air and counting the number of flies which regained postural control. We observed that heat shock alone in *para*^{ts1} flies does not cause ethanol resistance. Heat shock can substitute for control. **C**) *para*^{ts1} flies were age matched and divided into two groups. Heat shock

group received a 30 ºC heat shock for 5 hours (HS) on day 1. The second group received an ethanol sedation immediately followed by a heat shock incubation at 30 ºC for 5 hours (Ethanol+HS). After 24 hours a tolerance assay was performed where both groups received ethanol till they were sedated. Recovery assayed after switching the ethanol stream to air and counting the number of flies which regained postural control. We observed that heat shock did not block the acquisition of tolerance in *para*^{ts1} flies.
Statistical analysis is by the log rank test. This accounts for the differences in recovery of the whole population between the two curves (*$p<0.05$; **$p<0.01$). The p-values are indicated on the graphs if significant. Error bars are SEM for each data point.

Synaptic blockade due to *comt*^{tp7} does not block the acquisition of tolerance.

The *comatose* allele in Drosophila encodes for the NSF1 protein which is critical for vesicle fusion at the synapse and synaptic transmission. The allele, *comt*^{tp7} is a temperature sensitive allele. At the restrictive temperature of 35 ºC, these mutants experience conditional paralysis with underlying defects in synaptic vesicle fusion and exocytosis leading to accumulation of synaptic (7S) SNARE complexes. The phenotype does not instantaneously revert to normal when the flies are returned to the permissive room temperature. This contrasts with the very quick (within minutes) recovery seen with *para*^{ts1} or *shi*^{ts2} flies (Siddiqi and Benzer, 1976). Even after a very brief heat shock for 1-2 minutes, these flies require about 30 minutes to recover. This has been attributed to the time taken for dissociation of the 7S complexes that have accumulated at the synapse (Littleton et al., 1998). The flies eventually recover from paralysis and do not have any associated mortality or phenotypic defects over the course of their recovery and for the rest of their life span. Therefore, I modified the heat shock protocol in these flies and did only a 30 minute heat shock at the restrictive temperature of 35 ºC.

I performed the first control experiment, a tolerance assay with a control group versus an ethanol group, to show that *comt*^{tp7} flies could acquire tolerance to ethanol at the permissive temperature (Figure 5.3A). The flies were divided into two groups and given air (Control) and ethanol (Ethanol) on day 1. They were then returned to food and were assayed for ethanol tolerance on day 2. I observed that, at the permissive temperature, *comt*^{tp7} flies are capable of acquiring tolerance to ethanol sedation.

For the heat shock control experiment (to test for the effects of heat alone on ethanol tolerance in these flies), I used a heat shock protocol of 35 ºC for 30 minutes. Age

matched flies were divided into two groups which received air (Control) and a 35 ºC heat shock for 30 minutes (HS) on day 1. On day 2, both groups were subjected to the tolerance assay and recovery assayed for return of the righting reflex. I observed that *comt*^{tp7} flies became significantly sensitized to the effects of heat shock alone (Figure 5.3B). The fact that this effect was evident even after 24 hours (day 2) seemed to indicate that the exocytosis defect takes more than 24 hours to return to normal.

Following the controls, the third experiment was done with two groups of flies treated as follows. I compared flies that received a 35 ºC heat shock for 30 minutes (HS) to flies that received ethanol immediately followed by the 35 ºC heat shock for 30 minutes (Ethanol+HS) (Figure 5.3C). On day 2, the flies were subjected to the tolerance assay and recovery assayed every minute for return of the righting reflex. We observe that *comt*^{tp7} flies are able to acquire tolerance. This shows that blocking neurotransmission by interfering with synaptic exocytosis does not prevent the acquisition of tolerance in *comt*^{tp7} flies and suggests that tolerance is a cell-autonomous property of the nervous system.

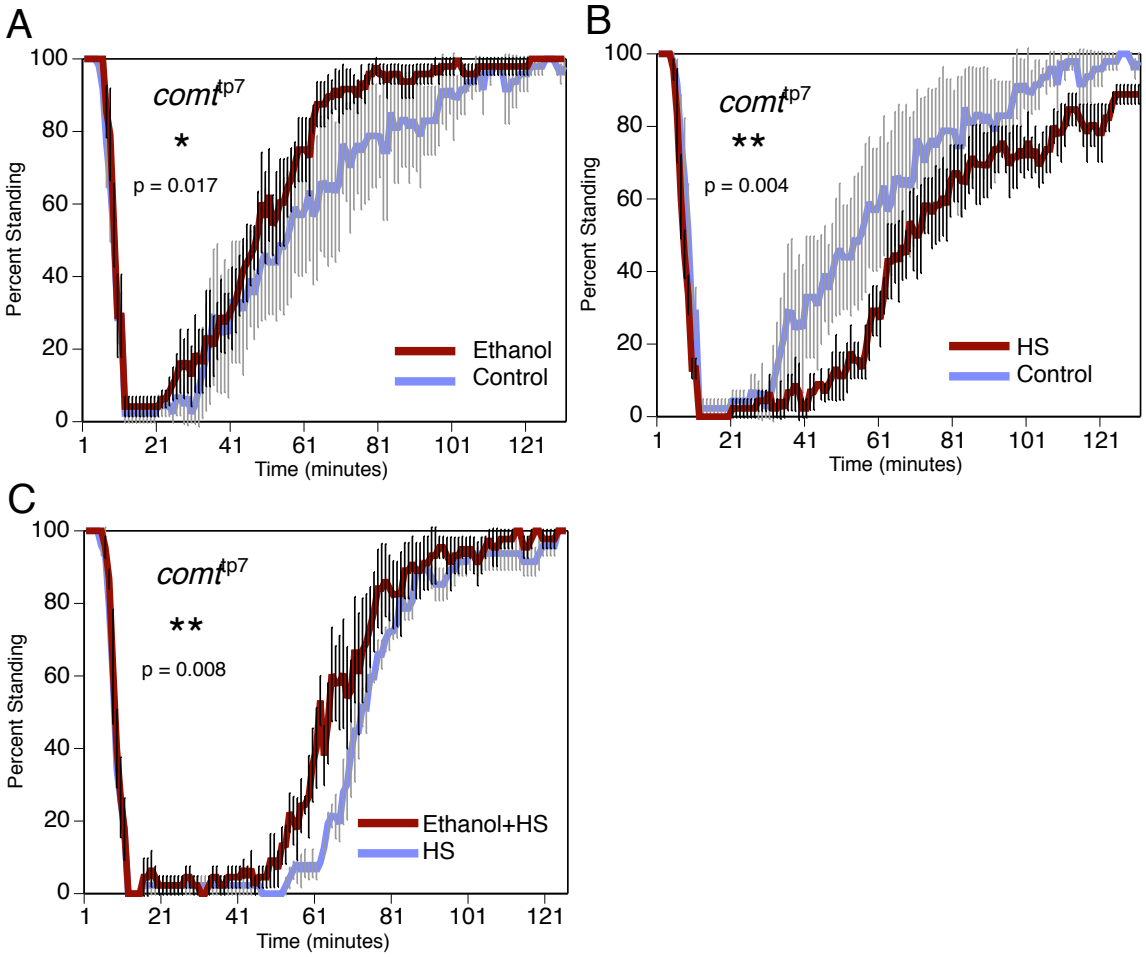


Figure 5.3. Acquisition of tolerance is not blocked by *comt*^{tp7}. **A)** *comt*^{tp7} flies were age matched and divided into two groups. Control flies received air (Control) and the ethanol group received an ethanol sedation (Ethanol) on day 1. After 24 hours a tolerance assay was performed where both groups received ethanol till they were sedated. Recovery assayed after switching the ethanol stream to air and counting the number of flies which regained postural control. We observed *comt*^{tp7} flies acquire ethanol tolerance at the permissive temperature. **B**) *comt*^{tp7} flies were age matched and divided into two groups. Control flies received air (Control) and the second group received a 35 ºC heat shock for 30 minutes (HS) on day 1. After 24 hours a tolerance assay was performed where both groups received ethanol till they were sedated. Recovery assayed after switching the ethanol stream to air and counting the number of flies which regained postural control. We observed that heat shock alone in *comt*^{tp7} flies causes sensitization to ethanol. **C**) *comt*^{tp7} flies were age matched and divided into two groups. Heat shock group received a 35 ºC heat shock for 30 minutes

(HS) on day 1. The second group received an ethanol sedation immediately followed by heat shock incubation at 35 ºC heat shock for 30 minutes (Ethanol+HS). After 24 hours a tolerance assay was performed where both groups received ethanol till they were sedated. Recovery assayed after switching the ethanol stream to air and counting the number of flies which regained postural control. We observed that heat shock does not block the acquisition of tolerance in *comt*^{tp7} flies.
Statistical analysis is by the log rank test. This accounts for the differences in recovery of the whole population between the two curves (*$p<0.05$; **$p<0.01$). The p-values are indicated on the graphs if significant. Error bars are SEM for each data point.

Drosophila *shibire* is involved in the acquisition of tolerance to ethanol.

A different allele of shibire, shi^{ts1}, does not acquire tolerance even at the permissive temperature.

Since I observed a difference in behavioral outcome among the three alleles, with shi^{ts2} blocking the acquisition of tolerance while $para^{ts1}$ and $comt^{tp7}$ did not, I wanted to rule out any direct effect *shibire* might have on ethanol tolerance. So, I tested a different allele of shibire, shi^{ts1}, for ethanol tolerance. This allele is also a temperature sensitive allele that paralyzes at the restrictive temperature of 30 ºC.

The first control experiment I performed was the tolerance assay at permissive temperature to see if shi^{ts1} can acquire ethanol tolerance at that temperature. These flies were divided into two groups and administered humidified air (Control) or saturated ethanol vapor (Ethanol) on day 1. On day 2, both groups received ethanol and were assayed for tolerance. I observed that shi^{ts1} flies do not acquire tolerance even at the permissive temperature (Figure 5.4). This indicates that the *shibire* gene may be involved in mediating acute ethanol tolerance in flies.

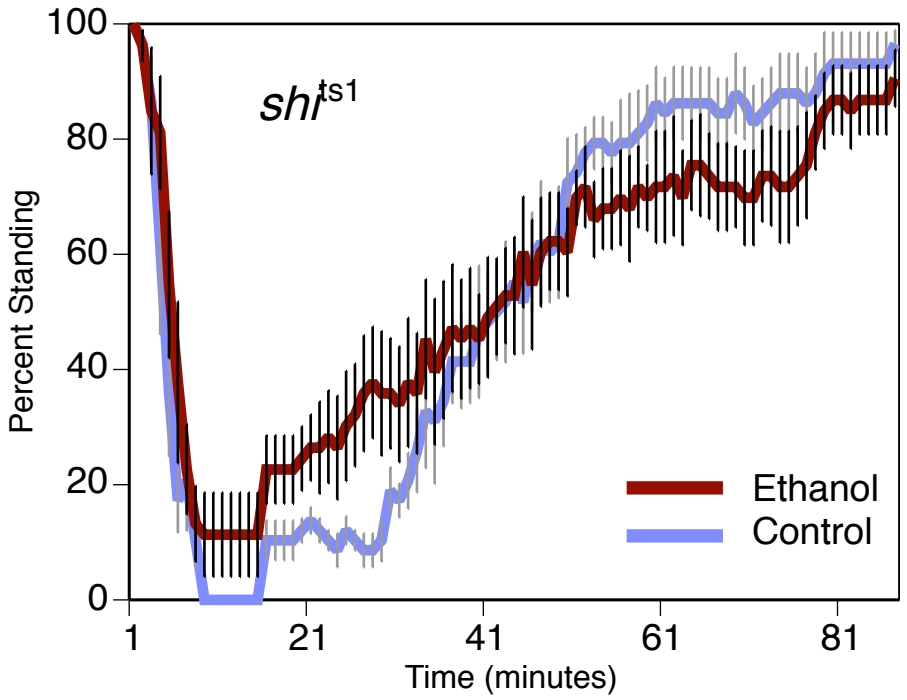


Figure 5.4. The *shibire* allele, *shi*^{ts1} does not acquire tolerance at permissive temperature.
A different allele of *shibire*, *shi*^{ts1} was tested for ethanol tolerance. Flies received air (Control) and saturated ethanol (Ethanol) on day 1. On day 2 both groups received ethanol and were subjected to the tolerance assay. Recovery assayed after switching the ethanol stream to air and counting the number of flies which regained postural control and recovery of the righting reflex. The flies do not acquire ethanol tolerance even at the permissive temperature.
Statistical analysis is by the log rank test. This accounts for the differences in recovery of the whole population between the two curves (*$p<0.05$; **$p<0.01$). The p-values are indicated on the graphs if significant. Error bars are SEM for each data point.

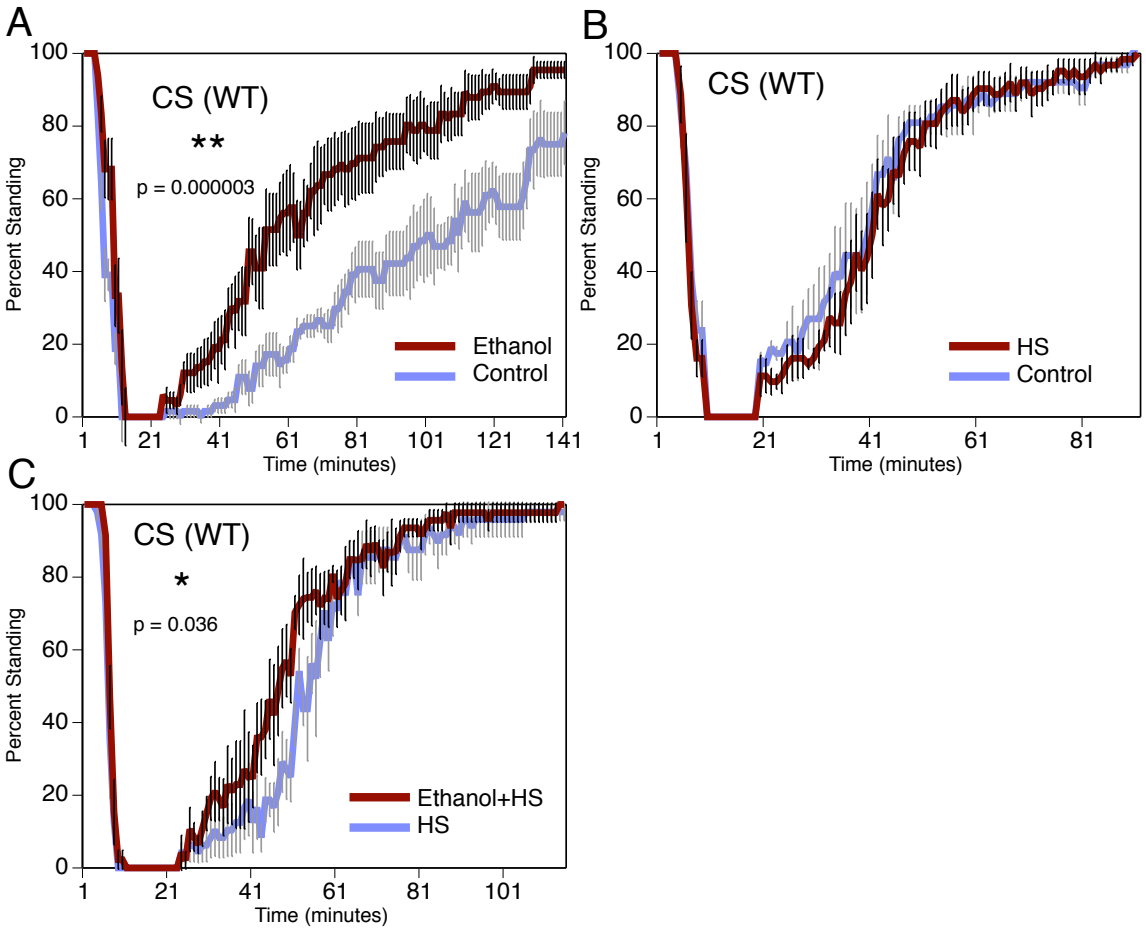


Figure 5.5. Canton S (CS) flies subjected to the heat shock protocol do not show any effects of heat on ethanol tolerance. **A)** CS flies were age matched and divided into two groups. Control flies received air (Control) and the ethanol group received ethanol sedation (Ethanol) on day 1. After 24 hours a tolerance assay was performed where both groups received ethanol till they were sedated. Recovery assayed after switching the ethanol stream to air and counting the number of flies which regained exhibited the righting reflex. We observed CS flies acquire ethanol tolerance at the permissive temperature. **B**) CS flies were age matched and divided into two groups. Control flies received air (Control) and the second group received a 30 ºC heat shock for 5 hours (HS) on day 1. After 24 hours a tolerance assay was performed where both groups received ethanol till they were sedated. Recovery assayed after switching the ethanol stream to air and counting the number of flies which regained postural control. We observed that heat shock alone in CS flies does not cause ethanol resistance or sensitization whatsoever. **C**) CS flies were age matched and divided into two groups. Heat shock group received a 30 ºC

heat shock for 5 hours (HS) on day 1. The second group received an ethanol sedation immediately followed by heat shock incubation at 30 ºC for 5 hours (Ethanol+HS). After 24 hours a tolerance assay was performed where both groups received ethanol till they were sedated. Recovery assayed after switching the ethanol stream to air and counting the number of flies which regained postural control. We observed that heat shock following ethanol sedation does not prevent ethanol tolerance in CS flies.
Statistical analysis is by the log rank test. This accounts for the differences in recovery of the whole population between the two curves (*$p<0.05$; **$p<0.01$). The p-values are indicated on the graphs if significant. Error bars are SEM for each data point.

DISCUSSION

Functional ethanol tolerance in Drosophila is a neuronal phenotype. To characterize this tolerance as an emergent or cell autonomous property of the brain, I blocked synaptic transmission in the nervous system to test whether I could prevent the acquisition of tolerance. Three neuronal temperature sensitive mutants (*shi*^{ts2}, *para*^{ts1} and *comt*^{tp7}) were used for blocking synaptic transmission in the nervous system. These mutants are well characterized temperature sensitive mutants that exhibit paralysis at restrictive temperatures and recover when moved to permissive temperatures.

Results from *para*^{ts1} (Figure 5.2) and *comt*^{tp7} (Figure 5.3) seem to indicate that inhibition of neuronal signaling after ethanol administration does not interfere with the capacity to acquire tolerance to subsequent ethanol sedation. Therefore we infer that ethanol tolerance is a property of specific neuronal circuits and is a cell autonomous property of the nervous system.

On the contrary, we observed that paralysis by a temperature-inducible block of *shi*^{ts2} could block the acquisition of tolerance in flies. Control experiments done with *shi*^{ts2} flies showed that these flies could acquire ethanol tolerance under permissive conditions (Figure 5.1A). The effect of heat shock alone resembled the *para*^{ts1} (Figure 5.2B) and wild-type (CS) (Figure 5.5B) phenotype with no significant differences from the control flies (Figure 5.1B). However, when subjected to the restrictive heat shock protocol, 30 ºC heat shock for 5 hours immediately after ethanol sedation on day 1, *shi*^{ts2} flies were unable to acquire tolerance on day 2. This is evident in figure 5.1C when the heat shock group (HS) is compared to the ethanol+heat shock group (Ethanol+HS). The Ethanol+HS curve is not significantly shifted from the HS curve. Since we inferred that

tolerance is a cell autonomous property of the nervous system based on the *para*^{ts1} and *comt*^{tp7} data, the most logical interpretation of these results could be that the *shibire* gene could have a direct role in mediating ethanol tolerance in flies.

In order to test this idea, I performed a tolerance assay with a different temperature sensitive allele of shibire, *shi*^{ts1}. These flies, unlike *shi*^{ts2}, did not show tolerance even at the permissive temperature (Figure 5.4). Thus, the overall conclusions from these experiments are that Drosophila *shibire* gene is important for rapid ethanol tolerance.

The allelic differences in *shibire* accounting for the different behavioral outcomes in the tolerance assay are not surprising given the fact that there are reports that *shibire* alleles exhibit subtle differences in their heat sensitivities and mutant phenotypes (Chen et al., 2002; Kim and Wu, 1990). Both *shibire* alleles that I tested, *shi*^{ts2} and *shi*^{ts1}, are point mutations in the crucial GTPase domain of *shibire* (van der Bliek and Meyerowitz, 1991). In our tolerance assay we see that *shi*^{ts2} flies acquire normal ethanol tolerance at the permissive temperature, and the loss of tolerance is only evident when we shift the flies to the restrictive temperature of 30 ºC (Figure 5.1). This is in contrast to the *shi*^{ts1} flies, which do not acquire tolerance even at the permissive temperature (Figure 5.4). This disparity could be due to the fact that the *shibire* alleles have been shown to have subtle differences in temperature kinetics. The recovery time from paralysis in *shi*^{ts1} is nearly 10 fold longer than *shi*^{ts2}. Also, the recovery time is strongly correlated with the length of the heat shock (Chen et al., 2002). Other documented differences include interactions of *shibire* with a mutation in NDP kinase in Drosophila, *awd* (abnormal wing discs), that decreases the availability of GTP, a critical substrate for DYNAMIN function. In the double mutant *shi;awd* the threshold for paralysis is almost 6 ºC lower in the case of

shi^{ts2}, but only about 1 ºC for *shi*^{ts1} (Chen et al., 2002). Such documented differences between the nature of the alleles seem to support our observations that there exists variability in ethanol responses among the two alleles.

Another important reason for the variability could be genetic background differences between the two alleles. To rule out such effects, we could setup back-crosses with the *shi*^{ts2} allele and wild type flies (Canton S) and re-isolate the allele. If the variability was due to background differences, we should expect the re-isolated *shi*^{ts2} to phenocopy the ethanol phenotype of *shi*^{ts1} and not acquire tolerance even at the permissive temperature.

Tolerance is a synaptic phenotype: roles played by shibire, homer and slowpoke

In mammalian systems, DYNAMIN III interacts at the post-synaptic density (PSD) with HOMER, the scaffolding protein SHANK and mGluR5 (Gray et al., 2003). SHANK is a scaffolding protein and has an SH3 domain that interacts with DYNAMIN and a EVH1 domain that interacts with HOMER. Thus, it can form a signaling complex at the synapse and regulate neuronal morphology and synaptic communication. HOMER has been implicated in drug responses in mammals (Szumlinski et al., 2004; Szumlinski et al., 2005). The *homer* gene in flies is responsible for ethanol tolerance (Urizar et al., 2007). *Homer* null mutants show decreased tolerance to the sedative effects of ethanol. Therefore any potential binding partner of *homer* could influence ethanol tolerance.

Flies have single genes encoding for *homer* and the homolog of SHANK in flies, *prosap*. Since flies have only one DYNAMIN homolog encoded by the *shibire* locus, it is possible that fly DYNAMIN could interact with HOMER and PROSAP at the synaptic membrane. An analysis of the sequence of the PROSAP protein reveals a conserved

EVH1 domain (PPXXF) that has been shown to functionally interact with HOMER in higher vertebrates (Figure 5.6). Drosophila PROSAP also has a DYNAMIN interacting SH3 domain. Thus, PROSAP could be functioning as an adapter molecule to functionally couple DYNAMIN and HOMER at the synapse. These interactions lend themselves to the testable hypothesis that an evolutionarily conserved signaling complex exists at the synaptic membrane that mediates ethanol tolerance .

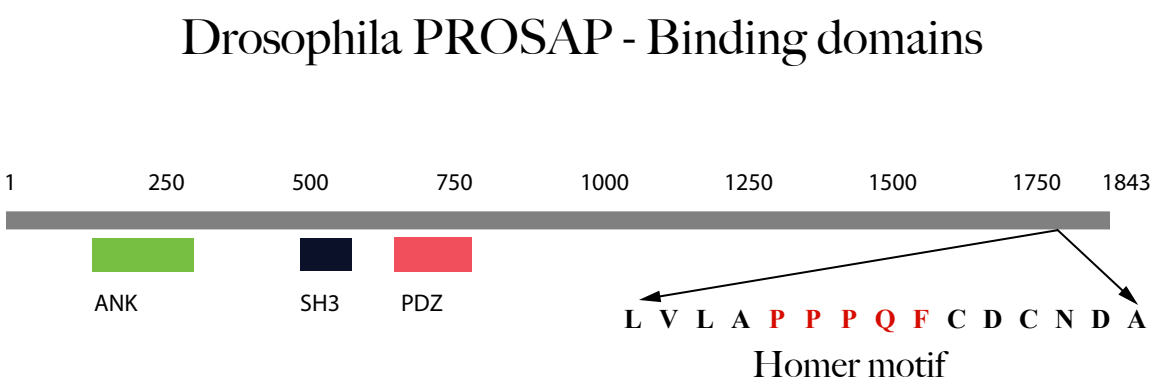


Figure 5.6. A Drosophila homolog of SHANK, PROSAP, has HOMER and DYNAMIN binding regions.
Drosophila encodes for a single homolog of the SHANK family of mammalian scaffolding proteins, the gene *prosap*. The PROSAP sequence has a putative HOMER binding motif and an SH3 domain that is capable of interacting with DYNAMIN. In mammalian systems SHANK has been implicated in organizing the post-synaptic density (PSD) along-with HOMER and SH3 interacting proteins such as DYNAMIN III. Fly DYNAMIN has an SH3 binding domain and could interact with PROSAP and HOMER forming a signaling complex at the synapse.

The *slowpoke* encoded BK channel in Drosophila co-localizes with voltage gated calcium channels at the synapse. One of the important functions of BK is rapid re-polarization of the membrane leading to an enhancement of firing frequency of CNS neurons. Work done in our lab has shown that following treatment with the anesthetic benzyl alcohol, the firing frequency of CNS neurons is increased following sedation. Artificial induction of *slowpoke* cDNA in B52H also seems to phenocopy this effect. We believe that this is a homogenous response to increase the net excitability of the circuit leading to behavioral tolerance (personal communication from *Alfredo Ghezzi*). The *shibire* gene regulates vesicle recycling at the synapse and is important for rapid synaptic neurotransmission in the brain. Thus, it is not surprising that proteins involved in synaptic transmission could be potential candidates mediating tolerance to benzyl alcohol and ethanol. If we consider *shibire* to be epistatic to *slowpoke*, then the double mutant $shi^{ts2};slo^{4}$ should also abolish the tolerance phenotype. However, when I performed this experiment the flies were very weak and had excessive convulsions rendering it impossible to assay for tolerance by scoring for recovery from sedation (data not shown). Nevertheless, it tells us that the double mutant phenotype is more severe than the individual phenotypes and indicates that the genes are probably epistatic to each other.

Chapter 6: Reduced neuronal signaling does not phenocopy tolerance

INTRODUCTION

Tolerance to benzyl alcohol and ethanol is mediated by an up-regulation of neuronal *slowpoke*. This up-regulation is a response to sedation by the drugs and is required for the acquisition of tolerance. Non-sedative doses of the drugs that only induce hyperactivity do not cause message induction or behavioral tolerance. This trend is observed with other hyperactive stimuli such as heat shock and non-sedative toluene exposure. *Slowpoke* message is down-regulated and behaviorally these flies exhibit sensitization when exposed to benzyl alcohol. On the other hand exposure to the sedative stimuli, cold and CO_2, up-regulates *slowpoke* message mimicking the induction we observe after drug induced sedation. This suggests a homeostatic regulation of the *slowpoke* gene to regulate neuronal excitability in response to either sedation or hyperexcitability to mediate drug tolerance (Ghezzi et al., 2004). When flies become hyperactive, they down-regulate *slowpoke* levels and exhibit sensitization to a subsequent exposure of the drug. Conversely a sedative event (including drug exposure on day 1) would increase *slowpoke* expression and cause tolerance to subsequent exposure to the drug.

To test if reduced neuronal signaling, without the presence of the drug on day 1, can phenocopy ethanol tolerance, I used three different approaches to reversibly induce non-anesthetic sedation in flies. The first approach was to use a temperature sensitive mutant to reduce neuronal activity. The voltage gated sodium channel mutation $para^{ts1}$ exhibits temperature sensitive rapid paralysis at 30 ^{0}C. When the flies are shifted to permissive temperatures of 22 ^{0}C (room temperature), they recover within minutes and

show no obvious behavioral defects (Siddiqi and Benzer, 1976). At elevated temperatures, there is a reduction in action potential generation due to reduced sodium channels in the neuron (Nelson and Wyman, 1990). It has been shown that the mutation eliminates local field potentials (LFPs) that are an indicator of brain activity (Nitz et al., 2002). At permissive temperature (22 ^{0}C), the spontaneously occurring LFPs in *para*^{ts1} resemble that of wild type flies, however at restrictive temperatures (30 ^{0}C), the LFPs were shown to be completely eliminated (Nitz et al., 2002). I used *para*^{ts1} to induce neuronal paralysis and test if reduced neuronal signaling can phenocopy drug sedation and cause tolerance to a subsequent exposure of the drug.

For the second approach, I used the non-solvent anesthetic CO_2 to induce sedation in wild type, Canton S (CS) flies. The sedation induced in flies exposed to CO_2 has been attributed to a reduction in the sensitivity of glutamate at larval neuromuscular junctions (NMJs) leading to reduced signaling and flaccid paralysis (Badre et al., 2005). Sub-anesthetic concentrations of CO_2 have also been postulated to depress spontaneous CNS activity in flies (Krishnan et al., 1996). CO_2 anesthesia is rapidly reversible and the flies do not have any associated mortality. I used a 30 minute CO_2 exposure to induce sedation and test if reduced neuronal activity can substitute for drug sedation and lead to behavioral tolerance .

In the third approach, cold sedation was used to induce rapid paralysis in wild type (CS) flies. At very low temperatures, flies exhibit a phenomenon known as chill coma wherein the membrane excitability is reduced due to changes in resting potential and muscle action potentials. The ionic transport due to the Na^+/K^+ pump is affected ultimately affecting ion channel function and membrane excitability (Hosler et al., 2000). Cold anesthesia is also rapidly reversible once flies are moved to room temperature with

no obvious phenotypic defects. I used cold (0 ^{0}C) to induce sedation in wild type flies and test if a reduction in neuronal activity can substitute for drug sedation and phenocopy behavioral tolerance.

RESULTS

Reduced neuronal signaling by *para*^{ts1} does not phenocopy ethanol tolerance.

I used the *para*^{ts1} sodium channel mutant to block action potentials and neuronal signaling in the brain. A tolerance assay performed on *para*^{ts1} flies showed that they can acquire tolerance at the permissive temperature. Age matched female flies were divided into two groups and one group received air on day 1 (Control) while the second group received a saturated ethanol stream on day 1 (Ethanol). On day 2, both groups were subjected to the tolerance assay. We observed that *para*^{ts1} can acquire ethanol tolerance (Figure 6.1A).

Blockade of neurotransmission was performed by administering a 5 hour heat shock at 30 ^{0}C (HS) on day 1. The control group of *para*^{ts1} flies received air on day 1 (Control). On day 2, both groups received ethanol and the recovery from ethanol sedation assayed. We observed that blocking neuronal activity in *para*^{ts1} flies did not confer resistance to ethanol sedation (Figure 6.1B). To control for the effect of the heat shock, I subjected wild type, Canton S (CS) flies to a 5 hour heat shock at 30 ^{0}C (HS) and compared them with flies that received air on day 1 (Control). I observed that the heat shock paradigm does not affect ethanol tolerance and the flies do not exhibit any phenotype due to heat alone (Figure 6.1C). The above data suggests that inhibiting neuronal signaling without exposure to the drug does not phenocopy tolerance. To acquire ethanol tolerance, flies require prior exposure to the drug.

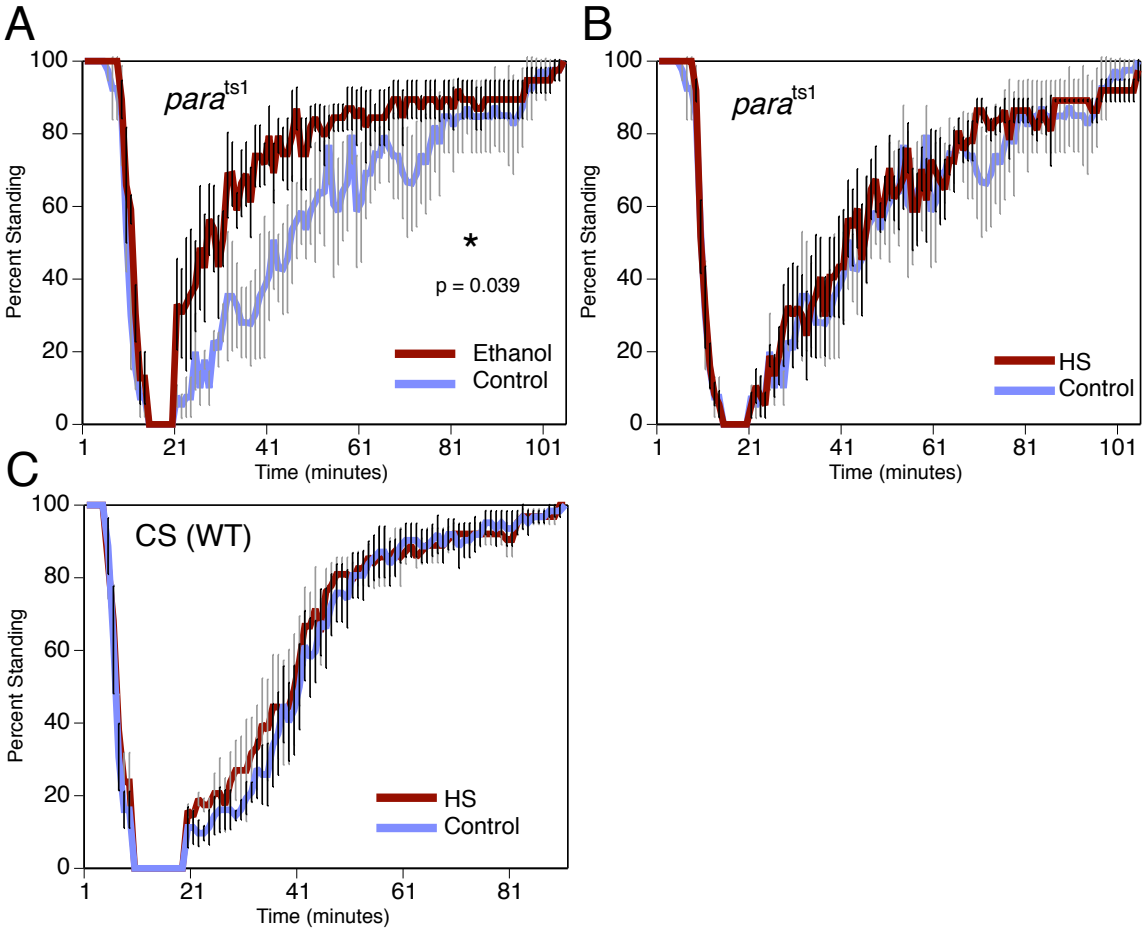


Figure 6.1. Inhibiting neuronal signaling in *para*^{ts1} does not phenocopy ethanol tolerance. **A)** *para*^{ts1} female flies were age matched and divided into two groups. The control and ethanol group received air (Control) and ethanol (Ethanol) vapor respectively on day 1. On day 2 both groups received ethanol and were subjected to the tolerance assay. Recovery assayed after switching the ethanol stream to air and counting the number of flies which regained postural control. *para*^{ts1} flies can acquire ethanol tolerance at the permissive temperature. **B)** *para*^{ts1} female flies were age matched and divided into two groups. The Control group received air on day 1 and the heat shock group received a 5 hour heat-shock at 30 ^{0}C (HS) on day 1. On day 2 both groups received ethanol and were subjected to the tolerance assay. Recovery assayed after switching the ethanol stream to air and counting the number of flies which regained postural control. Blocking neuronal transmission by inhibiting sodium channel action potentials does not phenocopy tolerance. **C)** CS flies were age matched and divided into two groups. The Control group received air on day 1 and the heat shock

group received a 5 hour heat-shock at 30 ^{0}C (HS) on day 1. On day 2 both groups received ethanol and were subjected to the tolerance assay. Recovery assayed after switching the ethanol stream to air and counting the number of flies which regained postural control. Heat shock did not affect ethanol tolerance in CS flies.

Statistical analysis is by the log rank test. This accounts for the differences in recovery of the whole population between the two curves (*$p<0.05$). The p-values are indicated on the graphs if significant. Error bars are SEM for each data point.

Sedation by the non-solvent anesthetic CO_2 does not mimic tolerance.

I used the non-solvent anesthetic CO_2 in wild type, Canton S (CS) flies to induce rapid sedation.

The tolerance assay was performed as a control experiment. I observed that CS flies can acquire ethanol tolerance. Age matched female flies were divided into two groups, one group received air on day 1 (Control) while the second group received a saturated ethanol stream on day 1 (Ethanol). On day 2, both groups were subjected to the tolerance assay and recovery from sedation assayed for return of the righting reflex. The ethanol treated flies recovered significantly faster from sedation (Figure 6.2A).

To induce CO_2 sedation, CS flies were age matched and divided into two groups, one group received air (Control) on day 1 while the second group (CO_2) received a constant stream of CO_2 for 30 minutes. CO_2 anesthesia is very rapid and flies sedate instantaneously. The recovery is also very quick with flies exhibiting normal movement within a minute of moving to fresh air. After 30 minutes, both groups were moved to food and tested for ethanol tolerance on day 2 (Figure 6.2B) . I observed that CO_2 sedation on day 1 did not cause flies to acquire resistance to a subsequent ethanol sedation. Sedation by CO_2 did not phenocopy drug induced tolerance.

Sedation by cold does not mimic tolerance.

Cold anesthesia is a rapid non-solvent sedation. Wild type (CS) flies were subjected to a 5 hour cold incubation and assayed for tolerance. The flies did not phenocopy drug induced tolerance.

CS flies were age matched and divided into two groups, one group received air (Control) on day 1 while the second group was incubated at 0 ^{0}C for 5 hours (Cold). Cold anesthesia is very rapid and flies pass out instantaneously with an equally quick recovery (within minutes) when shifted back to room temperature. After 5 hours, both groups were moved to food and tested for ethanol tolerance on day 2 (Figure 6.2C) . The flies did not acquire resistance to ethanol sedation suggesting that cold does not phenocopy ethanol tolerance.

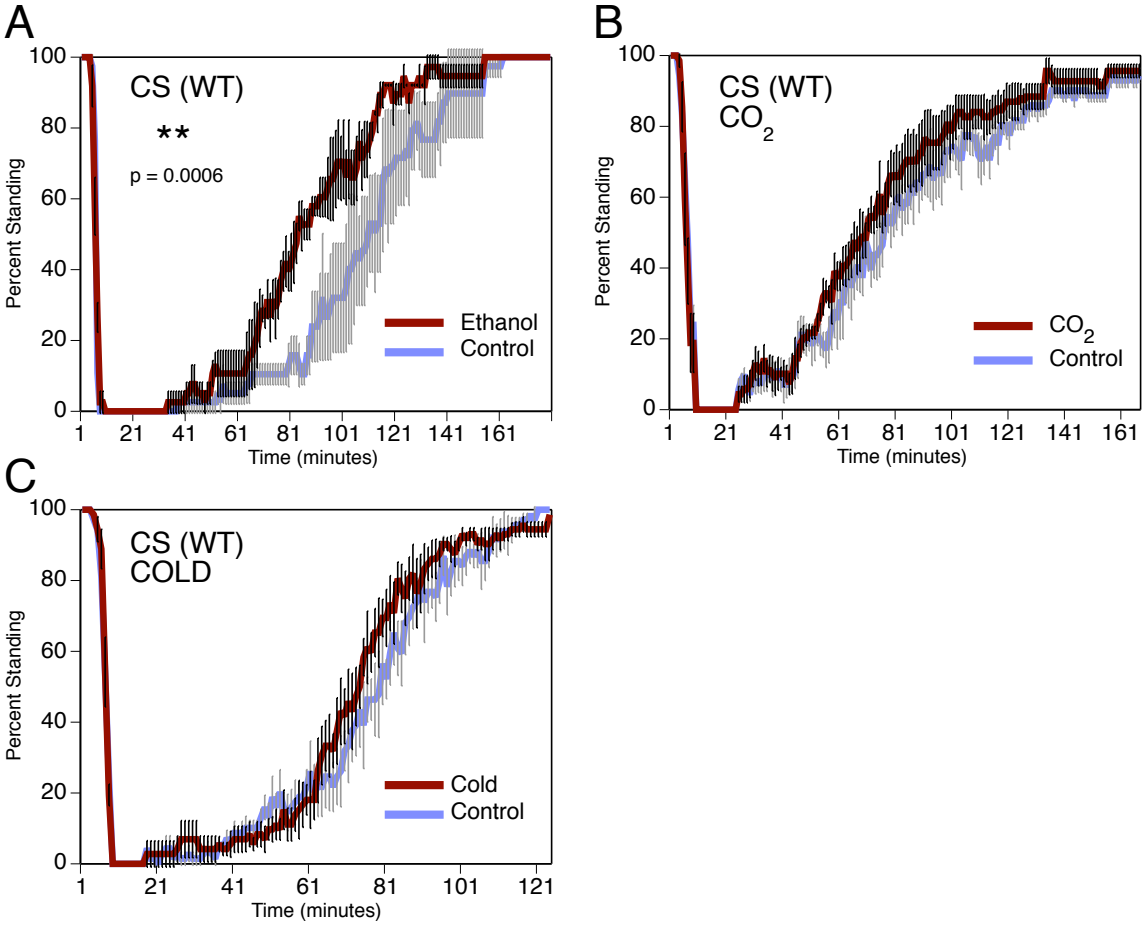


Figure 6.2. Non-solvent anesthetics CO_2 and cold do not phenocopy ethanol tolerance in CS flies. **A)** CS female flies were age matched and divided into two groups. The control and ethanol group received air (Control) and ethanol (Ethanol) vapor respectively on day 1. On day 2 both groups received ethanol and were subjected to the tolerance assay. Recovery assayed after switching the ethanol stream to air and counting the number of flies which regained postural control. CS flies were able to acquire functional ethanol tolerance. **B)** CS female flies were age matched and divided into two groups. The experimental group received a 30 minute sedation with CO_2 on day 1 (CO_2) while the control group received air (Control). On day 2 both groups received ethanol and were subjected to the tolerance assay. Recovery assayed after switching the ethanol stream to air and counting the number of flies which regained postural control. CO_2 induced sedation did not phenocopy ethanol tolerance in these flies. **C)** CS flies were age matched and divided into two groups. The Control group received air on day 1 and the cold group received a 5 hour cold incubation at 0 ^{0}C (Cold) on day 1. On day 2 both groups received ethanol and were

subjected to the tolerance assay. Recovery assayed after switching the ethanol stream to air and counting the number of flies which regained postural control. Cold sedation did not phenocopy ethanol tolerance in CS flies.
Statistical analysis is by the log rank test. This accounts for the differences in recovery of the whole population between the two curves (*$p<0.05$; **$p<0.01$). The p-values are indicated on the graphs if significant. Error bars are SEM for each data point.

DISCUSSION

Our hypothesis is that drug sedation homeostatically regulates neuronal excitability to cause tolerance. We had previously shown that treatments causing hyperactivity in flies such as heat and toluene administration tended to decrease *slowpoke* expression and cause sensitization to the drug. Treatments that decreased neuronal excitability tended to increase *slowpoke* expression, however, we did not observe tolerance to the drug (benzyl alcohol). Thus sedation without the drug, despite inducing *slowpoke*, did not phenocopy or mimic tolerance to a subsequent exposure to the drug. This could be attributed to the level of induction of the message. A 15 minute exposure to CO_2 and cold treatment was able to induce *slowpoke*, albeit to a much lower level than drug mediated induction (Ghezzi et al., 2004). So I used a stronger sedation with $para^{ts1}$ (5 hours), CO_2 (30 minutes) and cold (5 hours) to test whether drug is required on day 1 for tolerance. If the above treatments were able to mimic tolerance, it would suggest that reduced neural activity is sufficient to acquire tolerance to a drug. On the contrary, if the treatments did not mimic tolerance, it would suggest that some unknown consequence of the drug is required for tolerance to manifest.

The experiment performed with $para^{ts1}$ indicated that tolerance is a property of the drug and blocking neurotransmission by itself does not phenocopy the effect (Figure 6.1B). The *paralytic* locus in flies encodes for a voltage gated sodium channel α-subunit which is the predominant sodium channel in the brain of flies. The temperature sensitive allele $para^{ts1}$ is a mutant in which a heat shock causes a reduction in nerve action potentials concomitant with a sodium channel defect leading to rapid paralysis (Suzuki et al., 1971; Loughney et al., 1989; Elkins and Ganetzky, 1990). The defect has been

attributed to a reduction in the number of sodium channels. The phenotype is evident at higher restrictive temperatures leading to reduced capacity for repetitive activity and progressive action potential blockade (Nelson and Wyman, 1990). In order to mimic the sedation induced by ethanol exposure in flies, I used heat shock as a conditional stimulus in *para*^{ts1} flies. We see that reduced neuronal signaling alone does not predispose to ethanol tolerance in flies (Figure 6.1B).

In wild type, Canton S (CS) flies I observed the same result upon treatment with CO_2 and cold. CO_2 is a non-solvent anesthetic used commonly to sort flies; it induces a rapid paralysis which has been attributed to reduced glutamate sensitivity at the post-synapse (Badre et al., 2005). A 30 minute CO_2 sedation did not phenocopy ethanol tolerance in CS flies (Figure 6.2B). In the cold experiment, I incubated CS flies at 0 ºC for 5 hours to induce sedation on day 1. Cold lowers the basal metabolic rate and its block on neuronal transmission could be due to inhibition of ATPase dependent electrogenic pumps such as the Na^+/K^+ pump (Hosler et al., 2000). I observed that cold sedation did not mimic ethanol tolerance (Figure 6.2C).

Based on the above data we can conclude that exposure to the drug is necessary for tolerance. Reduced neuronal signaling by itself does not phenocopy or mimic drug induced sedation. Consistent with this conclusion is the fact that sedation by a different drug on day 1 can phenocopy tolerance. This phenomenon is called cross-tolerance. We have shown cross-tolerance between ethanol and benzyl alcohol in flies. Flies treated with ethanol on day 1 acquire tolerance to benzyl alcohol on day 2 compared to control flies and vice-versa (Cowmeadow et al., 2006). Cross-tolerance is also observed with barbiturates treatment on day 1 exhibiting cross-tolerance to both ethanol and benzodiazepines (Khanna et al., 1997; Khanna et al., 1998). Similarly glycine and

GABAA receptor function can be modulated by inhaled drugs of abuse such as TCE (1,1,1,trichloroethane), TCY (trichlorethylene) and toluene. Volatile anesthetics and ethanol target common residues shared with these inhaled drugs of abuse on the receptor channels (Beckstead et al., 2000).

Inhibiting neurotransmission does not phenocopy ethanol tolerance. Although the nervous system responds to reduced excitability by up-regulating *slowpoke*, the prior exposure of a drug is required to elicit tolerance.

Chapter 7: Methods

FLY STOCKS

Flies were raised on standard cornmeal/molasses/agar medium at 20˚C. Flies were kept on a 12 hour light/12 hour dark cycle with light starting at 9 am. When flies first started to eclose out of a food bottle, all the flies were cleared and new flies were then allowed to eclose over a 2 day period. They were then transferred to a fresh food bottle, and studied between 5 and 8 days later. For all experiments, unless otherwise noted, female flies were used.

Drosophila stocks used in this study were the following:

Wild-type stocks:

Canton S

Transgenic Stocks:

B52H (w^{1118} ;B52H ;slo^{4})

[In the B52H transgene, a heat inducible hsp70 promoter drives expression of a *slowpoke* cDNA].

854-OK107 (w*; P{GawB}OK107)

[GAL4 expressed in mushroom bodies].

7010 (w1118; P{Ddc-GAL4.L}4.3D)

[Expresses GAL4 in dopaminergic and serotonergic neurons].

6798 (w1118; P{Cha-GAL4.7.4}19B/CyO, P{sevRas1.V12}FK1)

[GAL4 expressed specifically in cholinergic neurons. Stock is over a balancer (CYO)].

6906 (w*; P{GawB}c309)

[GAL4 expressed in mushroom body, thoracic ganglion and eye-antennal disc].

Pan-Neuronal Gal4 driver:

6797 (w*; P{nrv2-GAL4.S}3; P{nrv2-GAL4.S}8)

[GAL4 expressed exclusively in the nervous system].

UAS-Slo (w^{1118},P{slo};slo^{4})

[The UAS-Slo construct was built by amplifying the *slowpoke* cDNA from B52H and subcloning into the pUAST vector].

Mutant Stocks:

slo^{4}; $para^{ts1}$; shi^{ts2}; shi^{ts1}; $comt^{tp7}$

CHROMIP ASSAY

About 1500 wild-type flies were either ethanol sedated or mock sedated for 5-7 minutes, and were allowed to recover in an ethanol free environment. Six, twenty-four and forty eight hours after sedation, flies were collected, frozen in liquid nitrogen, vortex decapitated and heads collected by sieving. Heads were cross-linked with 2% formaldehyde for 5 minutes and chromatin was solubilized and sonicated on ice 6 times 30 sec followed by 1 minute cooling on ice to produce fragments of ~600 bp with a sonic Dismembrator 250 (Fisher Scientific) as described by Orlando et. al. (Orlando V, 1997). Sheared soluble chromatin was stored at -80°C.

The chromatin immunoprecipitation assay was performed as described (ChIP kit # 17-295, Upstate Biotechnology) with minor modifications. One ml soluble chromatin (2 mg/ml) was adjusted to RIPA buffer and then pre-cleared with 50 ul salmon sperm DNA/ protein A agarose slurry for 1 hr at 4 °C to reduce nonspecific binding. Ten percent of the pre-immunoprecipitation lysate (100 ul) were saved as input for later normalization and processed with the eluted IP's beginning with the cross-linking reversal step. The polyclonal antibodies against acetylated H4 at K5, K8, K12 and K16 was used (catalog # 06-866, Upstate Biotechnology, NY) . Five microliters of antibody were added to each sample and incubated overnight at 4°C with gentle mixing. Immuno-complexes were recovered by adding 80 ul of the salmon sperm DNA/protein A agarose beads, incubating for 3 hr at 4 °C with rotation. The beads were sequentially washed three times in RIPA (140mM NaCl, 1mM EDTA, 10mM Tris-HCl, pH 8, 1% Triton X-100, 0.1% SDS, 0.1% Sodium Deoxycolate), twice in high salt buffer (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 500 mM NaCl, 20 mM Tris-HCl, pH 8), once in LiCl buffer (0.25 mM LiCl, 1% NP-40, 1% deoxycholate, 1 mM EDTA, 10mM Tris-HCl, pH 8) and twice in TE buffer, 10 minutes each. The cross-linking between histones and DNA was reversed by incubating at 65 °C overnight and DNA fragments were purified with phenol-chloroform extraction followed by acid ethanol precipitation. ChIP assays were performed three times with independent tissue samples.

Real-time PCR was performed using the ABI SYBR Green PCR protocol. Within the *slowpoke* transcriptional control region primers were designed to amplify ~200 bp fragments at the two neural promoters (C0, C1), at one muscle promoter (C2) and at five evolutionarily conserved areas (4b, 6b, cre1, scan2, 55b, c2 and c2c3). We used Cyc (*cyclophilin*) as the internal control. Primers sets are: C0 (5'-

ATCGAACGAAGCGTCCAG-3', 5'-CGACGCGCTCAAACG-3'), 4b (5'-GACCC GATGATAAAGTCGATGT-3', 5'-GCCAGTGACTGACTGACACACA-3'), 6b (5'-CCAGCAGCAATTGTGAGAAA-3', 5'-CGAAGCAGACTTGAAAGCAA-3'), C1 (5'-ACAAACCAAAACGCACAATG-3',5'-AATGGATGAAGGACTGGGAGT-3'), cre1(5'-GATGGGAAAGCGAAAAGACAT-3', 5'-CATGTCCGTCAAAGCGAAAC-3'), scan2 (5'-ATGCAATGAAGCGAAGAACC-3', 5'-CATTGCTATCCCTTCCCATC-3'), 55b (5'-ACCCAATTGAATTCGCCTTGTCTT-3', 5'CCCACTCTCCGGCCATCTCT-3'), C2 (5'-GCACTCGACTGCACTTGAAC-3', 5'-AATGAAAAAGTTCTCTCTGTGCAT-3') and Cyc (5'-TCTGCGTATGTGTGGCTCAT-3',5'-TACAGAACTCGCGCATTCAC-3').

All amplicons have differences in standard curve amplification slopes of less than 0.1. Amplifications were run in duplicate. Melting curves were used to detect nonspecific amplification. The relative amount of the acetylated-H4 histone was calculated by ΔΔCT method. Fold enrichment over control equals to 2^(CtInput – CtIP)experiment /2(CtInput – CtIP)control. The entire protocol has been repeated, in duplicate, a minimum of three times and the mean and SEM calculated. Significance was determined by Student's t-test.

ETHANOL ADMINISTRATION

Ethanol was administered to the flies in vapor form in an “inebriator”. Air entered the inebriator from a wall supply through Tygon tubing. The air supply was then split into two streams, each entering a flowmeter set to 15 ml/minute (one air stream was used for control treatments and the other was used for ethanol treatments). After exiting the flowmeter, each stream entered a water bubbler to humidify the air. A water bubbler consisted of a 250 ml Erlenmeyer flask with a #10 rubber stopper in it. The stopper had a hole in it just large enough to place a plastic 10 ml pipette through it. The pipette had the

ends cut off. The flask contained about 100 ml of distilled deionized water. One end of the pipette was submerged in the water. The air stream entered the water bubbler through tubing attached to the pipette, bubbled through the water and exited through tubing attached to the side arm of the flask. For the control stream of air, this tubing, led directly to a control treatment chamber. For the ethanol stream, this tubing led to a three way valve that could be switched to lead directly to two ethanol bubblers (Kontes part number 737610-0000), each containing 25 ml of 100% ethanol or that could be switched to skip the ethanol bubblers and to deliver the air stream directly to the treatment chamber. The bubblers were set in a 65˚C water bath to help ethanol evaporation. The ethanol bubblers were connected to each other with PFTE tubing. After exiting the bubblers, the ethanol stream entered a trap to collect any condensing ethanol. The trap resembled the water bubblers.

A treatment chamber consisted of two microfuge tube racks clamping together 6 standard plastic vials, containing the flies. The chambers contained a manifold to divide the incoming stream of air or ethanol vapor into six individual streams, each leading to one of the vials. Holes were drilled in the top microfuge rack to allow tubing from the manifold to enter the vials. A sheet of Viton® was used as gasket material to create an airtight seal between the vials and the top microfuge rack. A fine mesh was placed over the end of the tubing entering each vial to prevent flies from entering the tubing. Eight holes were poked in the bottom of each vial with a heated 25 gauge needle to allow air to exit the system.

TOLERANCE ASSAY

Sex and age-matched flies were assembled into 12 groups of 10 flies each. This includes six control and six experimental groups. For some experiments 10 (5x5), 8(4x4),

or 6 (3x3) groups were used. All flies were kept on a 12-12 day/night cycle.

Standard tolerance assay:

For the first treatment, flies were transferred from food vials to the treatment vials and placed in one of the two test chambers. One chamber was given a stream of air (control) and the other was given ethanol-saturated air (experimental). The ethanol stream was applied just until all the flies in the ethanol chamber were sedated. Sedated flies were scored as those which were lying on their backs or sides or those "face-down" with their legs splayed out in a non-standard posture. The ethanol stream was then switched to fresh air, and the flies were allowed to recover inside the chamber. When all the experimental flies had recovered, both groups, control and experimental, were transferred to food vials. At a later time point (~24 hours), all of the flies were returned to the inebriator and sedated with ethanol. For the control animals, this was their first ethanol exposure, while for the experimental animals it was their second exposure. The control and experimental groups of flies were interdigitated in the chambers to minimize any position effect within the testing apparatus. Ethanol was administered just until all flies were sedated, the ethanol was then withdrawn and replaced with fresh air. Both groups of flies remained in the chambers until all flies recovered. Tolerance was quantified during this second treatment by counting the number of flies recovered from sedation in each vial once every minute starting from the time ethanol was first applied to the time the flies recovered (sometimes a few flies take a long time to recover, and sometimes a few flies die). The results were graphed as the percentage of flies recovered from sedation over time for both the control and experimental groups.

Resistance Assays and Heat shock protocols:

For the B52H heat induction experiments (Chapter 2) the first ethanol sedation was replaced by a heat shock at 37 ºC for 30 minutes in glass vials without food. The controls were at room temperature in glass vials. After 6 and 24 hours, the tolerance assay was performed as described.

For the GAL4/UAS-Slo modified heat shock induction experiments (Chapter 4) the progeny from the crosses were incubated at 29 ºC vs 22 ºC for 3 days in food vials on a 12-12 day/night cycle. After three days the flies were removed from 29 ºC and allowed to equilibrate at 22 ºC for one day. On the fifth day the tolerance assay was performed as described above.

Testing for the temperature sensitive alleles was done as follows (Chapters 5 and 6):

para^{ts1} and *shi*^{ts2}:

HS: 5 hour heat shock at 30 ºC in glass vials (Cotton plugs were shoved into the vials and moistened with water so that the flies do not get dehydrated).

Ethanol+HS: Ethanol was administered on day 1 and as soon as the flies were sedated they were shifted to an incubator maintained at 30 ºC for 5 hours (Cotton plugs were shoved into the vials and moistened with water so that the flies do not get dehydrated). After that they were moved to food vials and left overnight. On day 2 a tolerance assay was performed as described.

comt^{tp7}:

HS: 30 minutes heat shock at 35 ºC

Ethanol+HS: Same as above except that after the flies were sedated they were moved to 35 ºC incubator for 30 minutes. They were returned to food afterwards and assayed for tolerance on day 2.

CO_2 and cold treatment (CS):

Canton S flies were treated with CO_2 for 30 minutes and then returned to food vials immediately after that. The controls were kept in empty vials breathing fresh air. On day 2 a tolerance assay was performed.

For the cold treatment CS flies were incubated at 0 ºC for 5 hours in glass vials. Cotton plugs were shoved into the vials and moistened with water so that the flies do not get dehydrated. The controls were kept at room temperature. On day 2 a tolerance assay was performed.

Quantitative Real-Time RT PCR

RNA was isolated from flies using a single-step RNA isolation protocol as described previously (Ghezzi et al., 2004) and quantified (NanoDrop Technologies).

Reverse transcription was set up with either gene specific or oligo-DT (Invitrogen®) primers as mentioned in the figure legend. 100ng of total RNA was used for a 20ul RT and a standard curve RT was set up with 25, 50, 100, 200, 400 ng of RNA. The protocol as given for SSII (Invitrogen®) was followed and at the end of the RT, the cDNA was diluted 1 in 5 by adding 80ul of ddH20. 5ul of the cDNA was used in a 25ul PCR reaction using ABI Sybr Green master mix protocol and gene specific primers in a 96 well plate. The PCR reactions were done in duplicates or triplicates and since we already had three groups for each treatment from the RNA preparation, the final yield was expressed as an average. Standard deviation and sem were calculated for n=3 and

normalized to an internal control, *cyclophilin* (CYC). Significance was calculated using the Student's t-test.

All the primer sequences used:

C1U- AAA CAA AGC TAA ATA AGT TGT GAA AGG A

C1RTL- GAT AGT TGT TCG TTC TTT TGA ATT TGA

C2U- GCT ATT TAT AAT AGA CGG GCC AAG TT

C2RTL- GGA AAT CCG AAA GAT ACG AAT GAT

CYCU- ACC AAC CAC AAC GGC ACT G

CYCRTL- TGC TTC AGC TCG AAG TTC TCA TC

UAS-SloU- ACC AAC ACA CAA GGT TCC G

UAS-SloRTL- CAC ACC ACA GAA GTA AGG TTC C

LacZU- CTG GCT GGA GTG CGA TCT TC

LacZRTL- GGG ATA GGT TAC GTT GGT GTA GAT G

Chapter 8: Conclusions

SUMMARY

Tolerance to ethanol is a neuronal phenotype that is mediated by the *slowpoke* Ca^{2+}-activated K^+ channel gene in flies. *slowpoke* message is up-regulated following a single sedative dose of ethanol and this transcriptional response coincides with behavioral tolerance. In mammalian systems, ethanol affects BK channel function post-translationally. It increases the open probability of the channel and modulates excitability of the circuit. In this study I have attempted to characterize *slowpoke* mediated ethanol tolerance at both the transcriptional and post-translational level.

slowpoke induction after benzyl alcohol and ethanol sedation is seen as early as 4-6 hours after a single brief drug sedation. Using the ChromIP assay, we demonstrated that the message up-regulation in response to benzyl alcohol involves increased H4 histone acetylation at the promoter of the gene (Wang et al., 2007). Acetylation is a positively acting post-translational modification (PTM) and facilitates *slowpoke* message induction. In order to characterize the acetylation state following ethanol sedation, I performed a ChromIP at different time points after sedation and quantified increased H4 acetylation at important conserved elements in the *slowpoke* promoter. This acetylation change has a spatio-temporal character with an early peak at 4b, 6b and 55b at 6 hours post ethanol administration. By 24 hours, we observed the increased acetylation shifting toward the neuronal promoter C0 and persistent at 6b. By 48 hours, the peak still remained focused at 6b. This persistent peak at 6b raises interesting questions. *slowpoke* message levels come back to baseline by 24 hours after sedation, however we still see a persistent acetylated state at 6b until 48 hours. If we are able to demonstrate a repressive

PTM at 24 and 48 hours at 6b then it will explain the message down-regulation. Repressive PTMs can occur on the same histone antagonizing a positive PTM such as phosphorylation or acetylation (Kouzarides, 2007). Acetylation promotes chromatin decondensation and makes the promoter available for other regulatory transcription factors. Thus we can have a molecular program unfolding at the promoter wherein ethanol mediated epigenetic changes activate transcription by acetylation at 55b and 6b. This can make the promoter accessible for a repressor to bind after message induction has peaked to homeostatically down-regulate it. Another important observation is that the acetylation in ethanol appears much earlier than with benzyl alcohol. The 6b peak in BA is maximal only at 24 hours while it appears at 6 hours in ethanol sedation (Figure 3.3). This could mean that ethanol is a stronger inducer of *slowpoke*. In that case an earlier time point, maybe 2 hours, could resemble the 6 hour BA pattern. Evolutionary sequence comparisons with different different species of Drosophila will reveal important conserved transcription factor binding sites which could be tested for promoter occupancy by using specific antibodies. Characterizing 6b and 55b will be important since both BA and ethanol show acetylation peaks at 6b and 55b. Deleting these sequences and testing for tolerance will be one of the priorities in the future.

The actions of ethanol affect the SLOWPOKE channel post-translationally in higher eukaryotes. The effects are pleiotropic with excitation in dorsal root ganglia neurons and GH3 pituitary cells and inhibitory in rat neurohypophyseal terminals. The channel can be phosphorylated by a variety of kinases PKA, PKC, PKG, CAMKII and phosphorylation is responsible for ethanol actions in bovine slowpoke channels. The Drosophila SLOWPOKE channel is also shown to be regulated by phosphorylation. We see a transcriptional up-regulation of the message following ethanol treatment which is

critical for the acquisition of tolerance. In this study, I show that tolerance also has a transcription-independent component. Since ethanol effects on BK are post-translational in higher vertebrates, the regulation in Drosophila could also be post-translational. The B52H flies I used for this study have a single *slowpoke* splice variant which makes it easy to target protein kinase substrates and delete them. This would facilitate identifying conserved residues critical for ethanol tolerance.

Mushroom bodies are critical structures in the fly brain involved in olfactory learning and classical conditioning (McGuire et al., 2001; Pascual and Preat, 2001). They are important regulators of the cAMP/PKA pathway. An allele of the *amnesiac* gene, *cheapdate*, shows increased sensitivity to ethanol and regulates mushroom body function (Moore et al., 1998). A mutant for the Ca^{2+}/calmodulin-sensitive adenylate cyclase, *rutabaga* and mutants for the catalytic subunit of cAMP-dependent protein kinase (*pka*-C1) show increased ethanol sensitivity and are expressed in the mushroom bodies (Wolf and Heberlein, 2003). Spatial induction of *slowpoke* in the mushroom bodies using the GAL4/UAS system phenocopies tolerance. This indicates that *slowpoke* could be acting upstream or downstream of a critical pathway regulating ethanol tolerance in the mushroom bodies. There are p[GAL4] lines which preferentially express GAL4 in specific lobes of the mushroom bodies that are implicated in different functions. This would help elucidate the precise role of *slowpoke* regulating mushroom body function to mediate ethanol tolerance.

The question does ethanol tolerance require synaptic communication, forms the basis of the emergent versus cell autonomous nature of tolerance. If blocking neuronal signaling also blocked the acquisition of tolerance, it would indicate that tolerance is as an emergent property of the nervous system as opposed to being a cell autonomous one.

This question was addressed by blocking neuronal signaling in the brain and then assaying for tolerance. Using two temperature sensitive alleles *para*^{ts1} and *comt*^{tp7}, I showed that tolerance is a cell autonomous property of the brain. In the course of this study, the temperature sensitive alleles of the fly dynamin gene, *shi*^{ts1} and *shi*^{ts2} were shown to affect the acquisition of tolerance. *shibire* mediated vesicle recycling at the synapse is important for rapid synaptic neurotransmission in the brain. Tolerance to sedative drugs is a homeostatic response of the nervous system to regulate excitability. SLOWPOKE modulates neuronal firing frequency thereby regulating neuronal excitability and DYNAMIN functions at the synapse to determine the efficacy of synaptic transmission. Thus, we have potential candidates that affect rapid synaptic signaling thereby regulating neuronal homeostasis to mediate tolerance to ethanol.

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