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Molecular mechanisms of alcohol tolerance in the fruit fly.

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Molecular mechanisms of alcohol tolerance in the fruit fly.

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Large conductance calcium-activated potassium channels have been shown to be potentiated by physiologically relevant acute doses of ethanol. Here I show that ethanol sedation increased transcription of the *slowpoke* gene, which encodes a large-conductance calcium-activated potassium channel, in the nervous system of the fruit fly *Drosophila melanogaster,* six hours after ethanol sedation. Twenty-four hours after sedation, neural *slowpoke* expression was decreased. Sedation with ethanol also induced tolerance that developed within four hours of sedation and persisted for at least seven days. *Drosophila* lacking *slowpoke* expression only in the nervous system were unable to acquire tolerance and flies which over-expressed *slowpoke* displayed resistance to the sedating effects of ethanol. The expression of several other ion channels was also increased six hours after ethanol sedation, however no other ion channel mutant tested showed a deficit in the capacity to acquire tolerance. Wild-type and *slowpoke* mutant flies showed no differences in ethanol metabolism following ethanol sedation that could account for the tolerance or lack of it. Therefore the *slowpoke* gene appears to play a unique role in the phenomenon of tolerance.

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CHAPTER 1 – INTRODUCTION

Alcohol is the one of the most widely used drugs in the world, second only to caffeine. It is also one of the most widely abused drugs (1). In the United States, almost seven percent of the population report drinking heavily, with heavy drinking defined as five or more drinks per occasion on five or more days in the last thirty days (2). The cost of this alcohol abuse is great, with a financial burden on our society of over $200 billion a year, for reasons such as lost productivity, medical costs, crime, and accidents (3).

The individual costs of excessive drinking are also great. Excessive drinking can lead to neuropsychological and structural deficits, as well as an increase in certain health risks, such as stroke, high-blood-pressure, cirrhosis of the liver, and cancer of the airway, digestive tract, and breast. People who are alcohol-dependent also have an increased risk of all types of mental disorders (4). In addition, over thirty percent of traffic fatalities involve alcohol (5).

Low to moderate alcohol use, on the other hand, can reduce the risk of cardiovascular disease, the leading cause of death of Americans, with moderate drinking defined as no more than two drinks a day for men, and no more than one drink a day for women, according to guidelines from the United States Department of Agriculture and United States Department of Health and Human Services (4). This may be related to the ability of alcohol to increase plasma high density lipoprotein-cholesterol levels (6).

However, people do not usually choose to drink based on health concerns. Stress reduction, mood elevation, increased sociability, and relaxation are the most commonly reported reasons for drinking (4). But moderate use far too often leads to abuse. This is probably because, like other addictive drugs such as cocaine, opioids, and amphetamines, alcohol acts on the mesolimbic dopamine system. This evolutionarily ancient system is

part of the motivational system that regulates responses to natural reinforcers, such as food, drink, sex, and social interactions. This system includes limbic structures such as the amygdala and hippocampus, which integrate emotion, reward, and behavior with motor and autonomic functions. Addictive drugs cause an increase in firing of dopamine neurons in the ventral tegmental area of the midbrain, resulting in an increase in dopamine levels in the nucleus accumbens and other areas of the limbic system (1, 7).

The molecular details of how exactly alcohol exerts its wide ranging effects on the nervous system are still under investigation, but much is now known. The effects of alcohol can be divided into two broad categories: the immediate effects on the nervous system that cause intoxication; and the long ranging effects which cause tolerance and perhaps addiction.

The immediate effects of alcohol include, at low doses, euphoria, hyperactivity, and relief from anxiety and inhibitions. As the dose increases, there is a diminished response to sensory stimulation, reduced physical activity, loss of coordination and balance, and depression of cognitive functions. At even higher doses there is drowsiness, hypnosis, anesthesia, and finally death due to respiratory failure (1).

The long-term effects of alcohol use include tolerance and addiction. Tolerance is defined as a reduced response to a drug with repeated exposure. In humans, tolerance can be due to several factors. There is a metabolic tolerance, meaning a greater ability to catabolize ethanol. There is also functional tolerance, which is an adaptation of the nervous system to the presence of the drug.

While tolerance almost always occurs with repeated exposure to ethanol, addiction does not. Addiction is the compulsive and uncontrolled pattern of drinking in spite of adverse consequences. The reason some people become addicted, and some do not, is not well understood. It is known that there is a genetic component to this phenomenon. There is

also a link between people with a low sensitivity to the effects of alcohol, and addiction (1).

Alcohol, or more specifically, ethanol, is a small, simple molecule that can easily diffuse across all biological membranes, including the blood-brain barrier. After it is consumed, it is rapidly and completely absorbed from the entire gastrointestinal tract, and becomes evenly distributed throughout all body tissues and fluids (1). The question remains though, how does a simple molecule like ethanol accomplish all its varied effects? It was once thought that, like other anesthetics, it mediated its effects by altering the physical properties of lipid bilayer membranes of cells. However, in the last two decades, there has been an overwhelming amount of evidence pointing to another mode of action of ethanol – its ability to interact with and modify the function of proteins of the nervous system (8).

It has been shown that ethanol can specifically and directly interact with many proteins of the nervous system, including ligand gated ion channels, voltage-gated ion channels, and second messenger proteins (9). The fact that the majority of proteins identified thus far are ion channels makes sense, as ion channels are primarily responsible for determining the excitability of a neuron, and ethanol has been shown to cause alterations in excitability of certain neurons and brain structures. Below is a brief summary of some, but certainly not all, or even most, of the known interactions between ion channels and ethanol. All of which tend to decrease the excitability of the nervous system.

Ethanol potentiates ion channels with an inhibitory function

$GABA_A$ RECEPTORS

The $GABA_A$ receptor has been the most extensively studied and implicated in the effect of ethanol on the nervous system. This ionotropic ligand-gated receptor recognizes the

neurotransmitter gamma-aminobutyric acid (GABA), the major inhibitory neurotransmitter in the brain. It is composed of five subunits surrounding an ion conducting pore. When the $GABA_A$ receptor binds GABA, it opens and allows the conductance of negatively charged chloride ions into neurons, causing decreased excitability of the cell. $GABA_A$ receptors are found at high density in the cerebral cortex, hippocampus, and cerebellum (1). The $GABA_A$ receptor has been shown to be potentiated by ethanol at intoxicating concentrations in many different preparations (10-14). However there are several types of $GABA_A$ subunits, and channels with different subunit compositions have been shown to have different sensitivities to ethanol (15).

GLYCINE RECEPTORS

Strychnine-sensitive glycine receptors (GlyR) are the major inhibitory receptor in the brainstem and spinal cord, but are also found in many other locations in the brain, such as the thalamus, hypothalamus, cerebellum, and cerebellar cortex. In the brainstem and spinal cord, GlyR control and coordinate locomotor activity, spinal reflexes and stretch reflexes. The structure of the GlyR is homologous to the $GABA_A$ receptor, it is composed of five subunits surrounding an ion conducting pore. When glycine binds to a GlyR, the receptor conducts chloride ions into a cell, decreasing its excitability (16). Like $GABA_A$ receptors, GlyR have been shown to be potentiated by acute ethanol exposure at intoxicating concentrations in many preparations (13, 14, 17, 18), and this potentiation is due to increased channel open probability (19).

NEURAL NICOTINIC ACETYLCHOLINE RECEPTORS

The nicotinic acetylcholine receptor (nAChR) is a ligand-gated ion channel in the same superfamily as the $GABA_A$ and glycine receptors. But unlike $GABA_A$ and glycine receptors, which conduct chloride ions, the nAChR conducts cations. Upon binding of acetylcholine, this channel opens and allows the flow of cations into the cell, increasing its excitability. However these channels are often found on GABAergic neurons, therefore by increasing their excitability, there is an increase in the release of GABA, an inhibitory neurotransmitter, resultying in an overall decrease in excitability. There are

several subtypes of nAChR: those found in skeletal muscles, and several neural subtypes (nnAChR), which can be classified based on their sensitivity to α-bungarotoxin. The α-bungarotoxin-insensitive nnAChR receptors have been found in small populations of neurons in the hippocampus, nucleus basalis, thalamus, superior colliculus, medial abenula, and interpenduncular nuclei. Low concentrations of ethanol have been shown to significantly potentiate these channels (20).

5-HT_3 RECEPTOR

A serotonin receptor has also been shown to be altered by ethanol. Serotonin has several types of receptors, both metabotropic and ionotropic. The 5-HT_3 receptor is an ionotropic receptor that allows the conductance of cations upon the binding of serotonin. This would generally be thought of as an excitatory effect, however, these receptors are often located in inhibitory interneurons, so activation of these receptors is generally inhibitory. In fact serotonin has been shown to inhibit hippocampal circuitry. These receptors are found in several locations in the peripheral and central nervous system, including inhibitory interneurons in the forebrain, cerebral cortex, and hippocampal gyri. Ethanol has been shown to potentiate these receptors by increasing their open probability (21).

GIRK

G-protein activated inwardly rectifying potassium (GIRK) channels are widely distributed throughout the brain and are normally activated by the binding of G-proteins released from metabotropic receptors, such as the m2 muscarinic, D2 dopaminergic, histamine, 5HT-1A, adensoine A1, $GABA_B$, and opioid receptors (22, 23). Once activated, these channels allow the influx of potassium ions, decreasing neuronal excitability. Acute ethanol exposure has been shown to activate both brain and cardiac GIRK channels at intoxicating concentrations without G proteins or second messengers present (24).

Ethanol inhibits ion channels with an excitatory function

NMDA RECEPTORS

About half the synapses in the brain are excitatory synapses that use glutamate as their neurotransmitter. There are three types of ionic glutamate receptors: NMDA, AMPA, and kainate. Binding of glutamate to these channels allows the influx of cations, increasing the excitability of the neuron. The NMDA receptor is particularly permeable to calcium, and is blocked by magnesium at resting potentials. Acute exposure to ethanol at intoxicating concentrations has been shown to potently inhibit these channels in a variety of neural preparations (25).

VOLTAGE-GATED CALCIUM CHANNELS

There are several types of voltage-gated calcium channels, which vary in their electrophysiological and pharmacological properties, as well as their expression patterns. The L-type channel has especially been implicated in the actions of ethanol. L-type channels are found many tissues, including the brain, heart, smooth muscle, and pancreas. They are found mainly in cell bodies and proximal dendrites. They are activated by depolarization, and in response allow the influx of calcium ions into neurons, increasing their excitability. Acute ethanol exposure has been shown to inhibit these channels due to a decrease in open probability (26).

Long term changes

The effects outlined above demonstrate some of the acute effects of ethanol that lead to a general depression of the nervous system. However, when ethanol is consumed chronically, the nervous system adapts to the presence of the drug in a manner generally opposite to its acute effects. This subject has not been as well studied, but generally it has been found that ion channels, which are potentiated by acute exposure to ethanol, are

down-regulated by chronic ethanol, and ion channels which are inhibited by acute exposure to ethanol, are up-regulated by chronic ethanol.

Chronic ethanol causes a decrease in the $GABA_A$ $\alpha 1$ subunit in the amygdala, and $\alpha 4$ subunit in the amygdala and nucleus accumbens (27). Chronic ethanol also causes the $GABA_A$ $\alpha 1$ subunit to decrease in the cerebral cortex, and does so by internalizing the receptor (28).

Chronic ethanol has been shown to up-regulate NMDA receptors. It has been shown that NMDA receptors were up-regulated following chronic ethanol exposure in hippocampal explants (29). Chronic ethanol also increased NMDA R1 and R2B polypeptide subunits in cortical neurons (30). This up-regulation of an excitatory channel leads to hyperactivity of the nervous system that sometimes results in seizures, and it has been shown that seizures caused by ethanol withdrawal were inhibited by an NMDA receptor antagonist (31).

Chronic ethanol also increases the density and function of neuronal L-type voltage-gated calcium channels. Chronic ethanol up-regulates L-type channels in rat inferior colliculus neurons (32) and in the neurohypophysis (33). This up-regulation has been shown to contribute to withdrawal hyperexcitability in hippocampal slices, and L-type channel antagonists have been shown to reduce withdrawal symptoms in rats (26).

Up-regulation or down-regulation of ion channels could occur by several mechanisms. The channel could be relocated within the cell, as sometimes happens to the $GABA_A$ $\alpha 1$ subunit. The channel could be modified in some way that affects function, such as phosphorylation. A very likely mechanism of regulation is a change in the transcription rate of the gene. In fact, the function of several transcription factors have been shown to be altered by ethanol. The transcription factor CREB (cyclic-AMP response element binding protein) has recently been strongly implicated (34).

CREB is a ubiquitously expressed transcription factor that recognizes and binds to CRE (cyclic-AMP response element) sites within the promoter regions of genes, enhancing their transcription. CRE sites have been found in many genes expressed in the nervous system, including neuropeptides, enzymes that synthesize neurotransmitters, and transcription factors, including CREB itself. CREB activates transcription when phosphorylated, which is done by PKA (cAMP-dependent protein kinase A) as well as several other kinases. PKA is activated by an increase in cAMP levels (34).

Acute ethanol treatment has been shown to increase levels of phosphorylated CREB (p-CREB) in several brain structures, while chronic ethanol treatment decreases p-CREB levels. It has been shown that acute ethanol treatment increased p-CREB levels in the nucleus accumbens of rats, and this effect lasted up to six hours, while chronic ethanol treatment decreased p-CREB levels (35). In mice, development of tolerance to the sedative and hypothermic effects of ethanol was accompanied by an increase in p-CREB levels in the cerebellum, hippocampus, and frontal cortex (36). Ethanol withdrawal, following chronic treatment, has been shown to decrease p-CREB, but not total protein levels, in the central and medial amygdala, but not in the basolateral amygdala (37). CREB phosphorylation was also lower in the shell of the nucleus accumbens in ethanol-preferring mice (38). Ethanol withdrawal, but not treatment, decreased p-CREB levels in the frontal, parietal, and piriform cortex in rats, but did not change overall protein levels. Ethanol treatment decreased both p-CREB and total CREB in the cingulate gyrus, while withdrawal increased total CREB (39). Ethanol consumption has been shown to reduce CREB phosphorylation in the nucleus accumbens of rats, specifically in the shell, but not the core of the nucleus accumbens, but did not change overall protein levels (40, 41). Finally, it has been shown that mice with reduced CREB expression have a higher preference for ethanol (42). These changes in activity of CREB in structures important to the ethanol response in the brain strongly implicate CREB as an important regulator of ethanol-induced transcriptional changes.

Slowpoke

Another ion channel which has recently been implicated in the actions of ethanol is the large-conductance calcium-activated potassium channel, Slowpoke. The *slowpoke* gene was first cloned from *Drosophila melanogaster* (43) and subsequently from mouse (mslo) (44) and human (hslo) (45, 46). The *slowpoke* gene codes for an α subunit of the Slowpoke channel. A complete channel is composed of four α subunit, with each α subunit composed of seven transmembrane domains (fig. 1). Their structure is very similar to other voltage-gated potassium channels, except for the presence of a calcium-sensing domain on the carboxy terminus of each subunit (47).

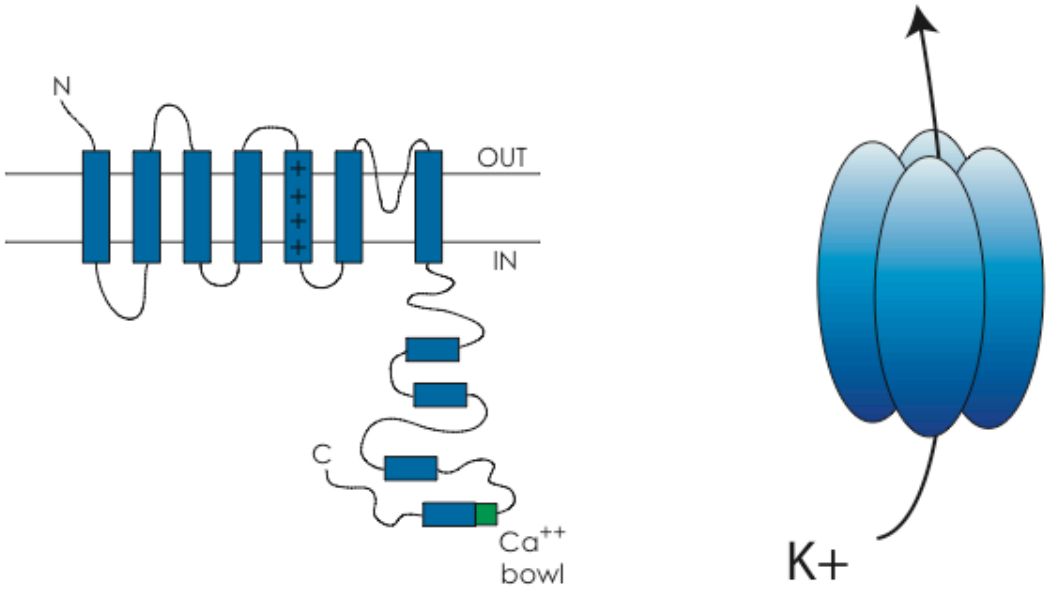


Figure 1. Slowpoke α subunit and assembled tetramer.

In vertebrates there are also ß subunits, which can associate with the α subunits, increasing their sensitivity to activation by calcium and voltage (48). Drosophila do not have ß subunits, but they do possess Slob, an accessory protein that binds to Slowpoke

channels, increasing channel activity (49). In Drosophila, Slob may play a role in circadian rhythms (50).

The activity of these channels integrate changes in both membrane potential and intracellular calcium. This channel will conduct potassium ions when there is both a depolarization of the membrane and the presence of calcium ions. These channels have a very high selectivity for potassium, as well as a very high conductance, between 100-300 pS, which is an order of magnitude larger than other potassium channels (51). Therefore they are also known as BK (big potassium) or Maxi-K channels. These channels are thought to regulate action potential duration and frequency. At normal, or resting, levels of calcium, this channel will not normally activate, as the voltage required is outside the normal potential fluctuations of a cell. However, when calcium enters the cytoplasm, either from intracellular stores, or through ligand-gated or voltage-gated calcium channels, the Slowpoke channel will activate in a voltage dependent manner. This channel is found at very high levels in axon terminals, where it is closely associated with voltage-gated calcium channels. This would allow rapid sensing of the increase in calcium levels following the invasion of an action potential into the terminal, which would allow the fine tuning of transmitter release. Association with ß subunits can cause these channels rapidly inactivate, which probably contributes to spike broadening observed during action potential bursts (52).

There is a single gene, called *slowpoke*, encoding this class of channels in both mammals and Drosophila. Transcripts from this gene undergo extensive alternative splicing to generate channels with different electrophysiological properties. Alternative splicing affects channel kinetics and calcium sensitivity (53-55).

These channels are widely distributed throughout both excitable and non-excitable cells (51). In the mammalian brain they are abundant in the hippocampus, cerebellum, thalamus, amygdala, neocortex, and olfactory cortex (52). They regulate action potential

shape, neuronal excitability, and transmitter release (51, 56-59). They are also abundant in smooth muscle, and endocrine tissue where they control smooth muscle tone and contractility, and neuroendocrine secretion (60).

Slowpoke channels have recently been found to play a role in cochlear tuning of hair cells. In the turtle and chick cochlea, these channels have been shown to determine the tonotopic resonant frequency of electrically tuned hair cells due to splice variants with different calcium sensitivities and kinetics as well as differential association of ß subunits (61-65). Slowpoke channels have also been shown to function in tuning of hair cells in the rat cochlea (66).

SLOWPOKE AND ETHANOL

The activity of the Slowpoke channel has been show to be altered by acute exposure to intoxicating concentrations of ethanol in a variety of preparations. Ethanol has been shown to potentiate Slowpoke channels in clonal pituitary (GH3) cells, and this was blocked by the presence of a PKC inhibitor (67). Ethanol also potentiates Slowpoke channels in rat neurohypophysial terminals, as well as mslo expressed in Xenopus oocytes (68). Ethanol also potentiates Slowpoke channels isolated from skeletal muscle T-tubule membranes and incorporated into planar lipid bilayer membranes, and this is due to increase in open probability (69). By contrast, ethanol failed to potentiate Slowpoke channels in rat supraoptic neuronal cell bodies (70). Ethanol actually inhibits Slowpoke channels in aortic myocytes due to an increase in mean closed time, and this was shown to not require the presence of ß subunits (71). In dorsal root ganglion neurons, ethanol increased Slowpoke channel activity and lead to a decrease in cell excitability and peptide release. By making chimeras of these channels, and those from aortic myocytes, which are inhibited by ethanol, it was determined that it is the core-linker region of the Slowpoke channel that determines whether it is inhibited or potentiated by ethanol (72).

In contrast to these acute effects of ethanol, chronic ethanol treatment in the rat neurohypophysis, where acute ethanol potentiates Slowpoke, has been shown to both decrease the potentiating effects of ethanol on the channel itself, as well as decrease channel density by internalization of the channel (33, 73).

SLOWPOKE TRANSCRIPTION

To further examine the role of the Slowpoke channel in the ethanol response, I have chosen to examine *slowpoke* gene expression in *Drosophila melanogaster* (fruit flies) following ethanol exposure. The work in my lab has previously focused on the transcriptional regulation of ion channel gene expression. The *slowpoke* ion channel gene, in particular, has been of great interest because of its complex developmental transcriptional regulation as well as its vast array of alternative splice variants, which lead to channels with different functional properties. In the last few years, the focus of the lab has expanded to include adult activity-dependent transcriptional regulation of ion channels. This led to the search for methods to alter the excitability of the nervous system of the fly and then examine whether transcription of any ion channel gene was changed as a result. Any change observed, could be either a homeostatic mechanism the fly employs to restore normal excitability, or could somehow be exacerbating the effects. For example, if a drug which in the short term decreased neuronal excitability, caused, in the long term, up-regulation of a voltage-gated calcium channel (excitatory), this could be considered a homeostatic mechanism. The up-regulation of an excitatory protein would be compensating for the depressive actions of the drug (although at times when there is no drug in the system this would also cause the nervous system to be hyperexcitable). If on the other hand this same drug caused a down-regulation of voltage-gated calcium channels, this down-regulation might be contributing to long lasting decreased excitability of the nervous system, or a ‘hang-over’ effect.

In Drosophila, Slowpoke channels are expressed in the nervous system, muscle, trachea and midgut. Transcription is controlled by a large upstream region covering 7 kb of genomic DNA. In this region there are five tissue specific promoters: two used for neural expression, two for midgut expression, and one for expression in both the muscle and trachea of the fly (74). The function of the remainder of the large 7 kb region is largely unknown, although several enhancers of muscle expression have been identified (75). The choice of promoter can alter the amino terminus of the channel, and there is also extensive alternative splicing of the mRNA that occurs, mostly in the carboxy terminus of the channel.

Slowpoke null mutant flies display several behavioral abnormalities. They do not fly properly, they have a defective mating song, and they display a behavior know as "sticky-feet" when given a strong stimulus such as light or heat, meaning they behave as if stuck to the ground when poked with a pencil, although they are not paralyzed.

I have chosen Drosophila to study the effects of ethanol on the transcripton of the *slowpoke* gene. Drosophila may not at first seem to be an ideal organism for ethanol research, when the majority of previous research has been on vertebrates, which would seem more relevant to the human condition. However Drosophila actually have several unique attributes and advantages which allow experimentation that is not possible at this time in other organisms. In general, flies are good experimental organisms for molecular or genetic research. They are simple to raise, and have a short generation time. They have well understood genetics, and many mutants are available, and their genome has been sequenced. Creating transgenic animals or targeted gene disruption is also relatively simple.

Flies are also one of the few organisms that normally encounter ethanol in their environment, in the form of fermenting plant matter. They have similar enzymes with which to metabolize ethanol as vertebrates. The behaviors elicited by intoxicated flies are

very similar to higher organisms, such as locomotor stimulation at low doses and sedation at high doses. They develop tolerance to, and show a preference for food containing alcohol. Drosophila also have a high degree of genetic conservation with humans. Most, if not all, the same proteins involved in neurotransmission, and proteins implicated in the ethanol response are conserved, such as $GABA_A$, NMDA, $5\text{-}HT_3$, adenosine, nAChR, multiple potassium and calcium channels, and second messenger systems. Flies also have a complex nervous system and are capable of complex behaviors, which can be manipulated and tested (76).

Flies have also been used successfully in the past as model animals to study the effects of ethanol. They had been used for years to study the alcohol dehydrogenase gene (77), and more recently have been used by the Heberlein lab to study aspects of ethanol induced changes in the nervous system. They have used Drosophila to identify mutants with altered sensitivity to the sedating effects of ethanol, which identified the *amnesiac* gene, a protein that activates the cAMP pathway (78). They have shown that dopamine is involved in the acute locomotor-activating effect, but not the sedating effect of ethanol (79). They have shown that mutants that cannot synthesize the catecholamine octopamine are unable to develop tolerance to ethanol (80). Finally, they have begun to determine which regions of the fly brain are important in ethanol-induced behaviors (81).

Recently Drosophila have also been used to show that silencing $GABA_BR1$ receptors by RNAi in adult Drosophila reduces ethanol-induced locomotor effects and enhancing $GABA_B$ with an agonist blocks rapid tolerance. This receptor, which is expressed in the central nervous system of the fly, has high homology to the mammalian $GABA_BR1$ (82).

I began my work interested in whether ion channel gene expression was altered in response to changed neural excitability. I chose to use ethanol intoxication as a means to transiently change the excitability of the nervous system. I have found that ethanol sedation of Drosophila increases transcription of the *slowpoke* gene, and that this increase

is limited to expression in the nervous system of the fly. This increase was seen six hours following ethanol sedation, but by twenty-four hours following sedation, *slowpoke* transcription had significantly decreased. The transcription of several other voltage-gated ion channels was also examined six hours following sedation, and all those examined showed an increase.

This increase in *slowpoke* transcription was also coincident with a gain of rapid tolerance to the sedating effects of ethanol. In wild-type flies the duration of sedation was significantly shorter in flies upon their second exposure to ethanol. Whereas, *slowpoke* null mutants were unable to acquire tolerance. Specifically, flies that lacked *slowpoke* expression only in the nervous system, but retained expression in other tissues, did not acquire tolerance. Conversely, flies that over-expressed *slowpoke*, displayed an inherent resistance to the sedating effects of ethanol upon their first exposure. Several other ion channel mutants were also tested, and all were able to acquire tolerance. I have also shown that this tolerance cannot be explained by a change in the metabolism of ethanol.

CHAPTER 2 – TRANSCRIPTION

Ethanol has been shown to induce changes in ion channel activity within minutes of application, which could account for the immediate intoxicating effects of ethanol. Ethanol can also cause long term changes to the nervous system, that last hours, days or even months, resulting in phenomena such as 'hang-over', tolerance or addiction. It has been shown that for several ion channels, the number of functional channels is altered by long term exposure to ethanol. Altering the number of functional channels can occur by several mechanisms, such as alteration of phosphorylation state, binding of accessory proteins, or localization. However, a common method to make changes that last for days or months is an alteration in the transcription rate.

The *slowpoke* gene has been shown to be an immediate target of ethanol. Its activity is generally potentiated by acute exposure to ethanol (69). To examine whether there is also a transcriptional response by this gene to ethanol, I have examined the transcription rate of the *slowpoke* gene in response to a single sedating dose of ethanol.

Administering ethanol to *Drosophila*

In order to test the effects of ethanol on the nervous system of the fly, a system first had to be devised to administer the drug. After much trial and error, an "inebriator" (fig. 2) was constructed that delivered ethanol to the flies in vapor form. Vapor administration of ethanol was used, rather than administration by injection or feeding, so that a sedating dose could be rapidly and equally delivered to a large population. Vapor could also be removed quickly and completely.

In the inebriator, air from a wall supply is first bubbled through water to humidify it. This is done to help minimize the dehydration that occurs during ethanol treatment.

Humidifying the air also helps standardize the amount of ethanol that can "dissolve" in the air. The air is then passed through a series of bubblers containing ethanol which are in a 65˚C water bath. The ethanol is heated to maximize evaporation and to saturate the air stream. I chose to use a saturated ethanol stream since it is mechanically the simplest concentration to reproduce. Because experimental and control animals are treated at the same time, small fluctuations in the ethanol dose are accounted for. Others who study the effects of ethanol in Drosophila have also chosen to standardize on an air-stream saturated with ethanol (78).

The air is then passed through a trap to collect any condensed liquid which would drown the flies. The mixture passes into a manifold that divides the stream of ethanol-saturated air into six tubes, each of which leads into the top of a plastic vial containing the flies to be treated. Holes in the bottom of the vials allow the air stream to escape. A parallel apparatus is used for mock or control treatments. In this second apparatus the air stream passes through a water bubbler, but bypasses the ethanol bubblers. During a single experiment 12 vials are available to treat flies, 6 in each chamber. Each chamber can have either fresh air, or ethanol-saturated air blown through it. Up to ten flies can be placed in each vial, for a maximum of 120 flies per experiment. When very large numbers of flies needed to be treated, the test chamber with the manifold and vials were replaced with a one liter plastic bottle with small holes poked in the bottom. The rest of the inebriator was unchanged. Hundreds of flies can be treated in this device.

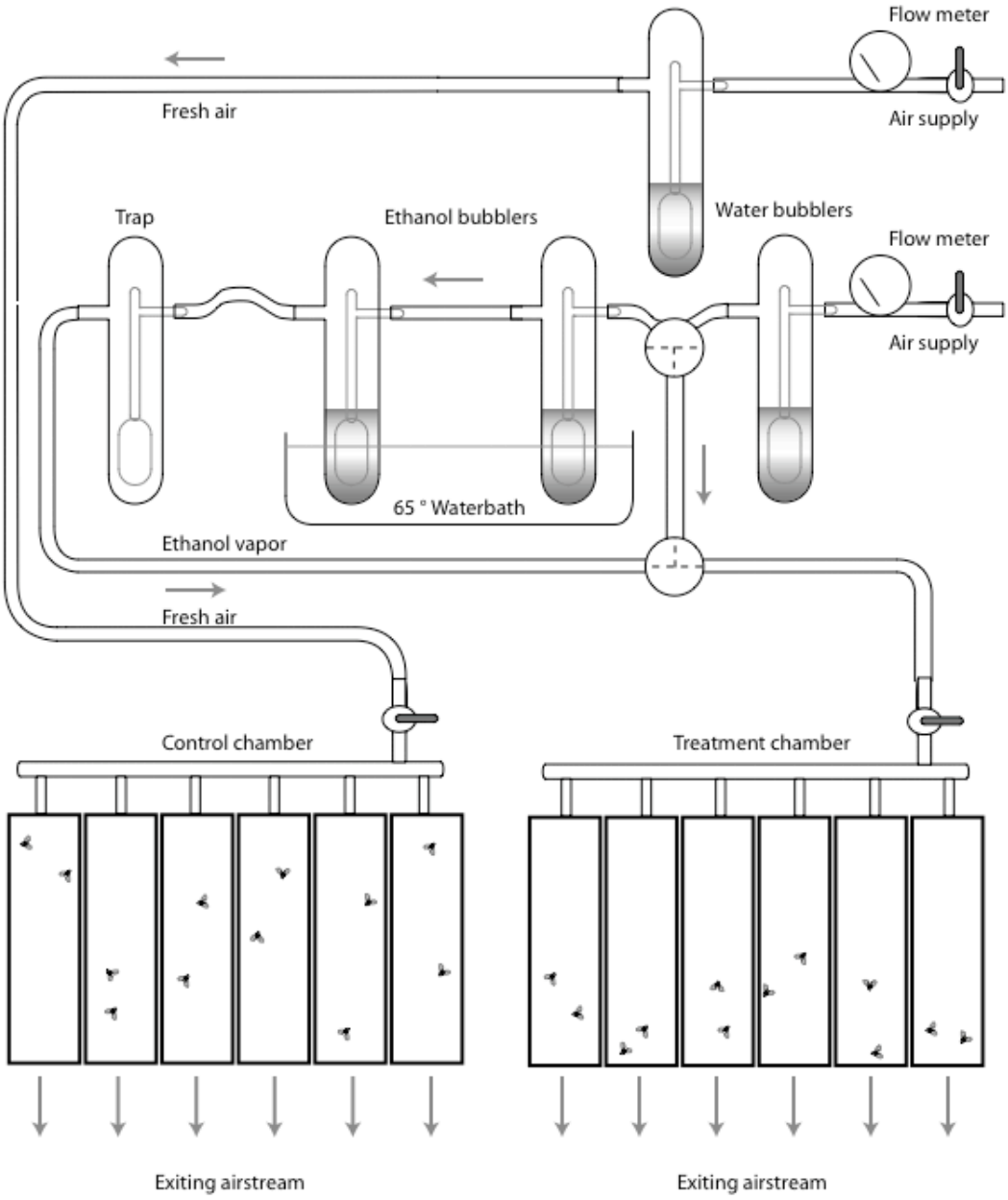


Figure 2. Inebriator used to administer ethanol to flies.

This device can deliver either ethanol-vapor or fresh air to vials containing flies.

During the first few minutes of ethanol exposure, the flies enter a hyperexcitable phase, in which they walk more, and at a greater speed. After a few more minutes their movement subsides, and eventually stops. After about ten minutes of ethanol exposure, flies become sedated. In my work, 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. These flies however, were not completely immobile. The legs of most of the flies which were lying on their backs, were twitching slightly. Recovery was scored when the flies stood upright. I chose not to use walking or climbing as an indication of recovery because following ethanol sedation flies appear to have a severe 'hang-over' and hardly move at all. This hang-over effect appears to last for several weeks.

During a typical treatment, flies are exposed to a stream of ethanol-saturated air just until the last fly has been sedated. At that point the ethanol stream is replaced with a stream of fresh-air to clear the vial of any residual ethanol vapor. After their return to fresh-air the animals require approximately 1 to 2 hours for the entire population to recover. Flies are said to have recovered from ethanol sedation when they stand upright. All flies, whether treated with ethanol or not, are left in their chambers until every ethanol-treated fly has recovered.

For all experiments, "matched" flies were used, meaning they were the same age (5-7 days), the same sex (female), had synchronized circadian rhythms, and had been collected from they same food bottles. They are not necessarily siblings, but were from the same generation and were closely related. The stocks used were not isogenic, (stocks that are inbred for many generations to make their genomes as identical as possible). This was an intentional decision made to ensure that any responses attributed to ethanol sedation would be sufficiently robust to be visible in flies with slightly different genetic backgrounds. Key experiments, repeated with different unrelated stocks, have shown that the described phenotypes are not dependent on a specific wild-type background. Females were chosen because early experiments showed that females were less likely to be killed

by any given ethanol treatment. To synchronize the circadian rhythms of the flies, all animals were all raised in a light/dark chamber, using a standard 12 hours of light and 12 hours of dark protocol; and, when the experimental protocol permitted, all ethanol treatments in all experiments were done at the same time of day.

Neuronal *slowpoke* is up-regulated in response to ethanol.

My original intent was to determine if *slowpoke* expression would respond to changes in electrical activity of the nervous system. I chose to use brief ethanol sedation as a means of altering neuronal activity. Subsequently, I have explored the idea that the ethanol-induced changes in neural activity are a homeostatic mechanism geared to restore normal neural activity. In these experiments, flies were sedated with ethanol, and their *slowpoke* mRNA levels measured. Age and sex matched w^{1118} flies were collected and divided into two groups (each group consisting of four vials of 7-8 flies each), one group was put in a test chamber and exposed to air saturated with ethanol vapor, while the other group was put in an identical test chamber but exposed only to fresh air to serve as a control. After all the ethanol-treated flies had been sedated, the ethanol vapor was replaced with fresh air and the flies were allowed to recover in the test chambers. Once all the sedated flies had recovered, both groups (ethanol-treated and control) were transferred to food vials. w^{1118} flies are a common laboratory strain of flies that are mutant for an eye pigment, but normal in other respects. They were chosen because they were the background stock used to generate lines of transgenic flies used in other experiments. However, key experiments have been repeated with other wild-type stocks.

Six hours after the beginning of the initial treatment, total RNA was isolated from both groups of flies. Quantitative real-time PCR was performed on RNA from both groups using primers specific for a neurally-expressed exon of *slowpoke* and an exon expressed only in the muscles. As an internal control, I also measured the relative abundance of mRNA from the *cyclophilin* gene. *Cyclophilin* mRNA is a common internal control used

by those interested in quantifying ethanol-induced changes in gene expression (83, 84). In our lab, we have also shown that *cyclophilin* mRNA abundance is not affected by sedation with other solvents such as benzyl alcohol (85). Levels of neurally expressed *slowpoke* mRNA were significantly higher in ethanol treated animals than in animals exposed only to air (fig. 3). Conversely, levels of muscle-specific mRNA did not show a significant change in ethanol treated animals compared to control animals (fig. 3).

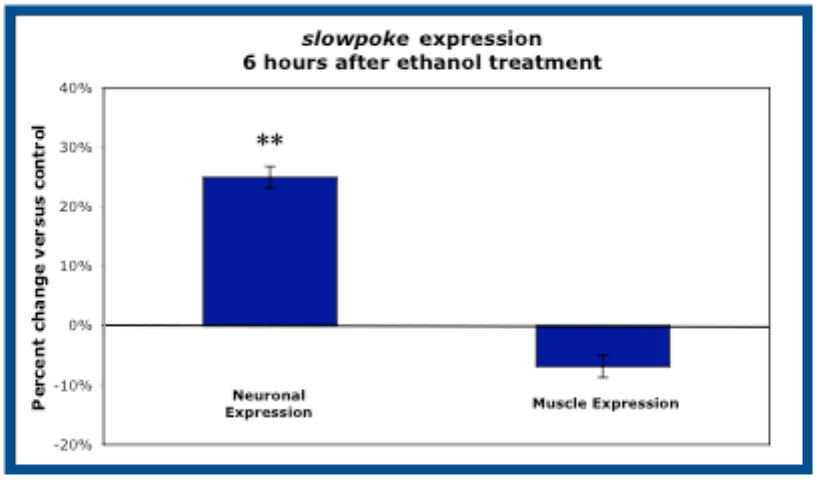


Figure 3. *slowpoke* expression 6 hours after ethanol treatment.

Levels of neurally expressed slowpoke mRNA, as measured by quantitative real-time PCR, were significantly higher in ethanol treated versus control animals. Levels of slowpoke mRNA expressed in the muscles was not significantly different in ethanol treated versus control animals.

To determine how long this increase in neuronal *slowpoke* expression persists, the previous experiment was repeated, however this time 24 hours was allowed to pass between the ethanol treatment and RNA isolation. Quantitative real-time PCR revealed that 24 hours after ethanol exposure, neuronal *slowpoke* mRNA levels had significantly decreased compared to control animals (fig. 4).

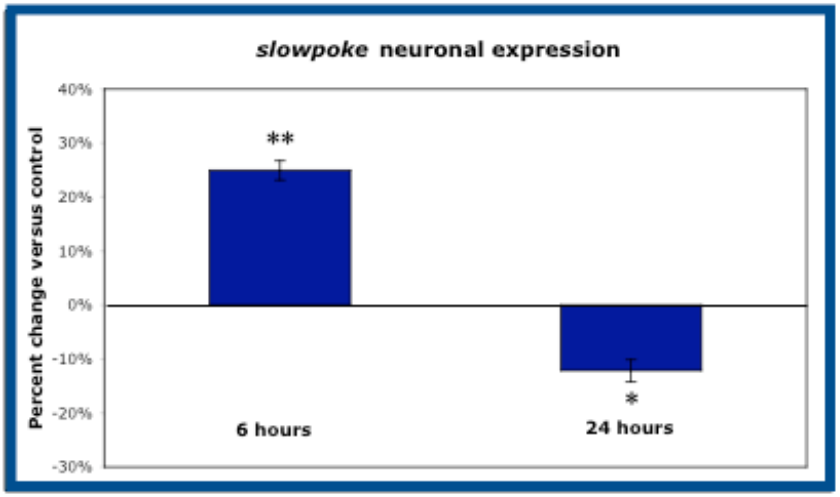


Figure 4. *slowpoke* expression in the nervous system at 6 hours and 24 hours after ethanol treatment.

Levels of neurally expressed *slowpoke* mRNA, as measured by quantitative real-time PCR, were significantly higher 6 hours after ethanol treatment compared to control animals. 24 hours after ethanol treatment, neuronal *slowpoke* mRNA levels were significantly lower in ethanol treated flies compared to control flies.

Increase in neuronal *slowpoke* mRNA is due to increased transcription.

The increase in *slowpoke* expression following ethanol sedation might arise from increased transcription initiation or from increased mRNA stability. To determine the origin in our system, I monitored the expression of a *slowpoke* reporter transgene called P3. The P3 transgene contains the portion of the *slowpoke* transcriptional control region necessary to reproduce the developmental and tissue-specific pattern of *slowpoke* in the nervous system of the fly (but it is not expressed in muscles, tracheal or midgut cells) (74). In the P3 transgene, the neuronal portion of the *slowpoke* transcriptional control region drives expression of the *ß-galactosidase* (*ß-gal*) reporter gene. The transcription unit is terminated by an SV40 polyadenylation signal (fig. 5). The stability of an mRNA is largely determined by its 3' end, so it is unlikely that events that alter the stability of

the *slowpoke* mRNA would also alter the stability of an mRNA with such different sequence.

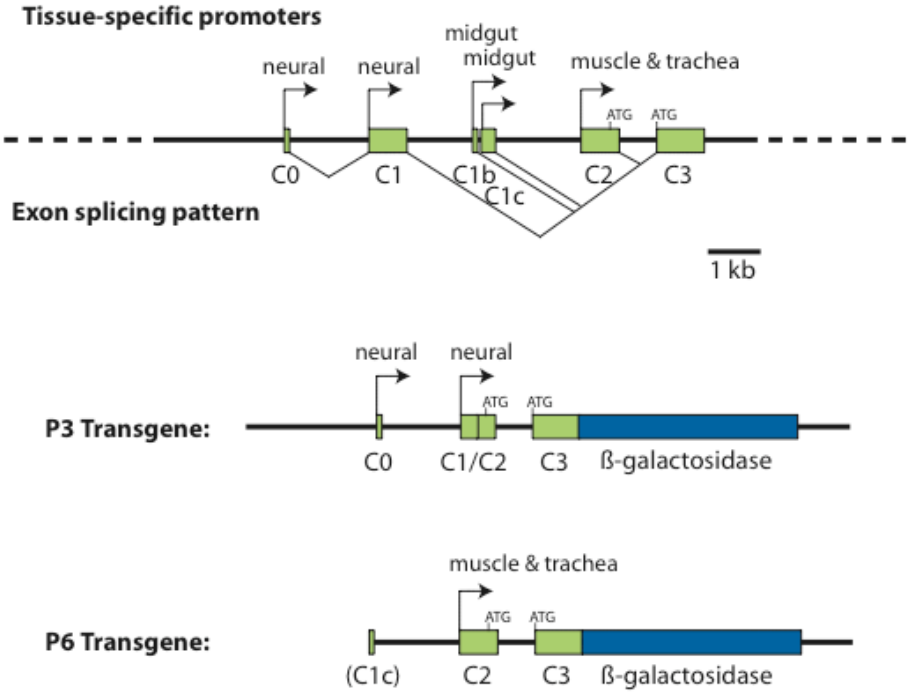


Figure 5. Transcriptional control region of the *slowpoke* gene and two transgenes. The P3 transgene deletes the midgut and muscle promoters. The P6 transgene deletes the neural and midgut promoters.

P3 transgenic flies were generated from a w^{1118} line of flies, therefore they have two normal copies of the *slowpoke* gene, in addition to two copies of the transgene. The transgene also has a functional copy of the eye pigment gene that is mutant in w^{1118} flies.

Matched P3 flies were collected and divided into two groups, each group consisting of 150 flies each. One group was put in a test chamber and exposed to air saturated with ethanol vapor, the other group served as the control group and was put in an identical test

chamber but exposed only to fresh-air. Once all of the ethanol-exposed flies were sedated, the ethanol was replaced with fresh-air and the flies were allowed to recover in the test chamber. After all ethanol treated flies had recovered, the experimental and control groups were transferred to food vials, 10 flies per vial. At various time points following the initial ethanol treatment, three vials of the ethanol-treated and three vials of control P3 flies were assayed for *ß-gal* activity and protein levels. *ß-gal* levels were normalized against protein levels. Flies that had been treated with ethanol showed an increase in *ß-gal* activity compared to control animals. The magnitude of the increase in *ß-gal* activity peaked at approximately 21 hours after treatment (fig. 6).

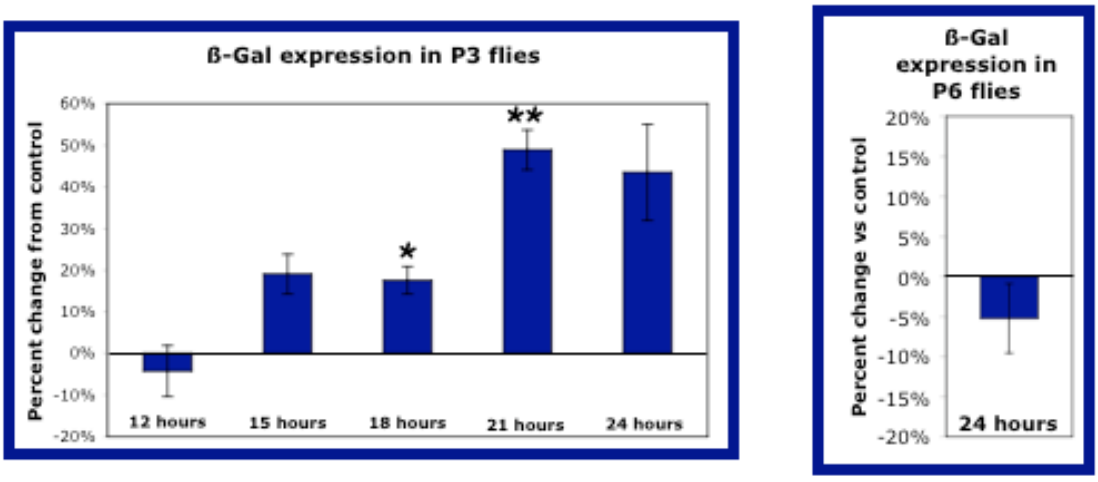


Figure 6. Expression of *ß-gal* in P3 and P6 transgenic flies.

Measurements taken at various time points after ethanol treatment compared to control flies. Each value is an average of three ethanol treated samples divided by the average of three control samples. Expression of *ß-gal* in P3 flies increases over time, peaking at around 21 hours. No significant change was seen in *ß-gal* levels in P6 flies.

Perhaps the increase in expression from the P3 transgene was due to some unknown interaction between this enzyme or its substrate, and ethanol. To eliminate this possibility, another line of transgenic flies called P6 was also treated with ethanol and

assayed for *ß-gal* activity. The P6 transgene contains the portion of the *slowpoke* transcriptional control region necessary to reproduce the developmental and tissue-specific pattern of *slowpoke* in the musculature and trachea of the fly (but not the nervous system or midgut). This fragment of DNA also drives expression of a *ß-gal* reporter gene in a w^{1118} background (fig. 5) (74). Matched P6 flies were collected and divided into two groups, one group was put in a test chamber and exposed to air saturated with ethanol vapor, the other group was put in an identical test chamber but exposed only to fresh air to serve as a control. Once all the flies exposed to ethanol were sedated, the ethanol was replaced with fresh air and the flies were allowed to recover in the test chamber. After all ethanol treated flies had recovered, both groups of flies were transferred to food vials, 10 flies per vial. Twenty-four hours following the initial ethanol treatment, three vials of the ethanol-treated and three vials of control P6 flies were assayed for *ß-gal* activity and protein levels. *ß-gal* levels were normalized against protein levels. Flies which had been treated with ethanol showed no significant difference in *ß-gal* activity compared to control animals (fig. 6).

Levels of other ion channel mRNAs also increase 6 hours after ethanol treatment.

The mRNA levels for several other ion channels were also measured in flies six hours after ethanol treatment. *Paralytic* (*para*) is a voltage-gated sodium channel. *Cacophony* (*cac*) is a voltage-gated calcium channel. *Shaker* (*Sh*), *seizure* (*sei*), *shaw*, and *shal* are all voltage-gated potassium channels. In all cases, levels of mRNA had increased in flies exposed to ethanol compared to control flies (fig 7).

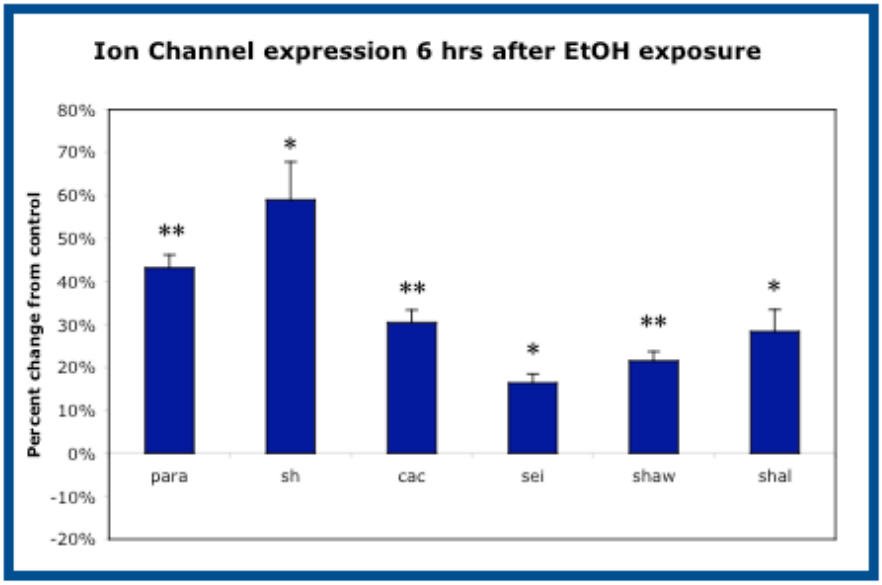


Figure 7. Expression of different ion channel genes 6 hours after ethanol treatment. Levels measured by quantitative real-time PCR, values given are the average of four ethanol-treated fly RNA samples relative to the average of four control fly RNA samples. Expression of all channels increased compared to control flies.

Conclusions

Quantitative real time PCR has demonstrated that *slowpoke* expression in neurons, but not muscles, increases 6 hours after ethanol sedation. It has also shown that 24 hours after ethanol sedation, neuronal *slowpoke* levels have decreased below control levels. The evidence from P3 transgenic flies also demonstrated an increase in neuronal *slowpoke* expression following ethanol sedation. This increase in the product of a reporter gene whose mRNA has a different sequence than that of the native *slowpoke* mRNA supports the interpretation that the increase is due to a change in transcription initiation and not a change in mRNA stability. The apparent delay in the increase in *ß-gal* expression in P3

flies (peaked around 21 hours) compared to *slowpoke* mRNA levels might be due to the time it takes for translation of the message and the fact that the *ß-gal* protein probably has a longer half-life than *slowpoke* mRNA and so can accumulate over a longer period of time. The evidence from the P6 line of transgenic flies, showing no change in expression from a muscle-specific *slowpoke* transgene, demonstrates that the increase seen in P3 flies was due to increased transcription, and not some interaction between ethanol and *ß*-galactosidase or its substrate.

I have shown that there is an alteration in *slowpoke* transcription, but there are several key questions that remain unanswered. How is this change in transcription accomplished? Why are these changes occurring and what are the actual consequences of the changes?

The alteration in *slowpoke* transcription due to ethanol sedation indicates that there must be transcriptional control elements, present in the *slowpoke* transcriptional control region, that mediate the response. More specifically, these elements must be within the 5 kb of transcriptional control region carried in the P3 transgene. These elements must respond either specifically to ethanol, or to the change in overall excitability of the nervous system caused by ethanol sedation. Related work in the laboratory has demonstrated that many different treatments, all of which alter the excitability of the nervous system, lead to a change in expression of *slowpoke* (85). Therefore I suspect that the increase in *slowpoke* expression is due to the altered excitability of the nervous system, though the exact mediating mechanism is not known.

The initial increase and subsequent decrease in *slowpoke* expression is very interesting as it is reminiscent of the changes in phosphorylation state of the CREB transcription factor. Acute ethanol exposure causes an increase in phosphorylation of CREB that lasts for up to six hours, while chronic ethanol exposure causes a decrease in phosphorylated CREB, levels (35). In fact, CRE sites have been found within the *slowpoke* transcriptional control region. There are also other transcription factors associated with ethanol that have

different time courses of activation following ethanol exposure. C-Fos levels rise and fall rapidly within six hours of ethanol exposure, while ΔFosB levels rise and fall much more slowly (7).

As to why these changes in transcription are occurring, they could be a homeostatic mechanism, meant to restore appropriate excitability to the nervous system after depression by ethanol. This would be the case if the Slowpoke channel had an overall excitatory effect on the cells in question. The transcriptional changes could also be exacerbating the effects of ethanol, causing further depression of the nervous system. This would be the case if the Slowpoke channel had an overall inhibitory effect on the cells in question, which is what you would suspect of a typical potassium channel. However Slowpoke is an unusual potassium channel. It has been shown to be both excitatory and inhibitory in different cell types and under different circumstances.

In several preparations Slowpoke has been shown to have an inhibitory role. Drosophila *slowpoke* null mutants have been shown to have broader action potentials in neurons (86) and adult longitudinal flight muscles (87). This also demonstrates that this current is responsible for repolarizing action potentials in these tissues. It has also been shown that mutations in *slowpoke* causes action potentials to occur in Drosophila larval muscle, a tissue that normally does not have action potentials, only passive propagation (88).

However slowpoke has also been shown to be excitatory in some situations. Drosophila *slowpoke* null mutants have been shown to have decreased transmitter release at the neuromuscular junction (89), and mutating *slowpoke* or blocking it with charybdotoxin virtually eliminated all heartbeat in the Drosophila cardiac pacemaker (90). An explanation for the mechanism of *slowpoke* mutations leading to inhibition of the nervous system is that by broadening action potentials, the time between action potentials would be longer, therefore in repetitively firing neurons, the frequency of firing would be reduced by mutating the *slowpoke* gene.

The majority of evidence points to Slowpoke playing an excitatory role in the nervous system of the fly. I suspect that the increase in *slowpoke* transcription leads to an increase in Slowpoke channels, and this is done as a homeostatic mechanism to counteract the depressive effects of ethanol. The decrease in *slowpoke* transcription seen at twenty-four hours may seem counter to this hypothesis, however there are several possible explanations for this decrease. Perhaps the initial increase in transcripiton causes the production of many Slowpoke channels, and perhaps these channels have a very long half life, so that a further increase in transcription is unnecessary to maintian an increased level of channel proteins. Perhaps there is just a brief pulse of increased Slowpoke, and this alters the excitability of the cells in such a way that a cascade of other changes occurs, maintaining other homeostatic changes.

In order to truly understand what is occuring, more information must be gathered. For instatnce, we do not yet know in which specific neurons transcriptional changes are occurring, nor do we know if the increase or decrease observed is the same in every neuron involved. We also do not know which splice variants of *slowpoke* are involved, as different splice variants have different electrical properties. Finally, we do not know if this change in transcription actually leads to a change in functional channels.

Work along these lines has recently been done in the rat hypothalamic-neurohypophysial system, which is responsible for release of oxytocin and vasopressin. Acute ethanol exposure has been shown to reduce the amount of hormone released, while chronic ethanol treatment leads to tolerance to this effect, meaning that further ethanol does not reduce hormone release (33). Acute exposure to ethanol has been shown to potentiate Slowpoke in this system, due to alterations in the open probability. While chronic ethanol treatment leads to an decrease in Slowpoke channel density (91). This potentiation of Slowpoke channels, starts to decrease within 12 minutes of ethanol exposure and remains decreased over 24 hours. The decrease in Slowpoke channel density starts to occur at 6

hours, and remains decreased over 24 hours. This decrease in density is due to shift in channels away from membrane, to interior, and is not due to change in overall expression (73).

Of course any explanation of the consequences of ethanol on the nervous system will have to account for all the changes that occur in a given neuron or system, as all molecules in a neuron effect one another to some degree. I could not examine all genes involved in the fly nervous system, but I did examine changes to several other ion channels, which would presumably play a prominent role in neuronal excitability.

Quantitative real-time PCR revealed an increase in the expression of all other ion channel genes tested six hours after ethanol sedation. Six other ion channel genes were examined: a voltage-gated sodium channel gene(*paralytic*), a voltage-gated calcium channel gene (*cacophony*), and four voltage-gated potassium channel genes (*Shaker*, *seizure*, *shaw*, and *shal*). All channel genes showed an increase in mRNA levels, however it is unknown whether this is due to a change in transcription or mRNA stability, or in what cell types it occurs, or whether it leads to more functional channels. The reason for this general increase in other ion channels is not clear, as some are excitatory and some are inhibitory. Perhaps it is part of a general increase in channel density in order to increase the responsiveness of the nervous system, which could counteract the general depression of the nervous system caused by ethanol.

Finally, although I have seen changes in other ion channels, it is unlikely that this is as significant as the *slowpoke* changes. As discussed in the next chapter, *slowpoke* null mutant flies, as well as several other ion channel mutants, were tested for an altered response to ethanol. Only *slowpoke* mutants showed a difference from wild-type animals. This seems reasonable, as the conductance of the Slowpoke channel is much larger than any of the other channels tested, up to 50 fold in some cases (92). Therefore a change in Slowpoke would probably have a much greater impact on the excitability of a neuron.

CHAPTER 3 - TOLERANCE

I was first interested in whether ion channel gene expression was modulated by ethanol sedation and I subsequently became interested in the phenomenon of tolerance. Tolerance is defined as a reduced response to a drug after repeated exposure to that drug (7), and rapid tolerance is produced by a brief single exposure to a drug. Drosophila have been shown to acquire rapid tolerance to the sedating effects of ethanol (80). Using my treatment protocol described in the last chapter, I looked for a manifestation of tolerance which could be easily measured. For this purpose I chose duration of sedation. I observed that wild-type flies acquire rapid tolerance to ethanol, meaning that flies, which had previously been sedated with ethanol, remained sedated for a shorter period of time during their second treatment compared to naïve flies. I had previously observed that sedation resulted in an increase in the abundance of mRNAs of a number of ion channel genes. However, the *slowpoke* channel gene was unusual in that animals carrying mutations in this gene were unable to acquire tolerance, and flies with excess *slowpoke* expression were resistant to ethanol's effects.

Tolerance Protocol

Sex and age-matched flies were assembled into twelve groups of 10 flies each. This includes six control and six experimental groups. For the first treatment, the flies were transferred from food vials to the treatment vials and placed in one of the two test chambers in the inebriator. One chamber was given a stream of air (control) and the other was given ethanol-saturated air (experimental). The air stream was applied until all the flies in the ethanol chamber were sedated. The ethanol stream was then switched to fresh air, and the flies were allowed to recover inside the chambers. When all the experimental flies had recovered, both groups, control and experimental, were transferred to food vials. At a later time point (4 or 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 until all flies were sedated, the ethanol was then withdrawn and replaced with fresh air. Both groups of flies remained in their chambers until all animals had recovered. Tolerance was quantified during this second treatment by counting the number of flies recovered from sedation in each vial once every minute from the time the ethanol was first applied. The recovery time varies from animal to animal. Some flies wake up in 2 or 3 minutes while a few flies take many, many hours to wake up. Occasionally, a few flies are killed by the treatment (fig. 8). All tolerance tests were done at both 4 hours and 24 hours between treatments. Results were the same in each case.

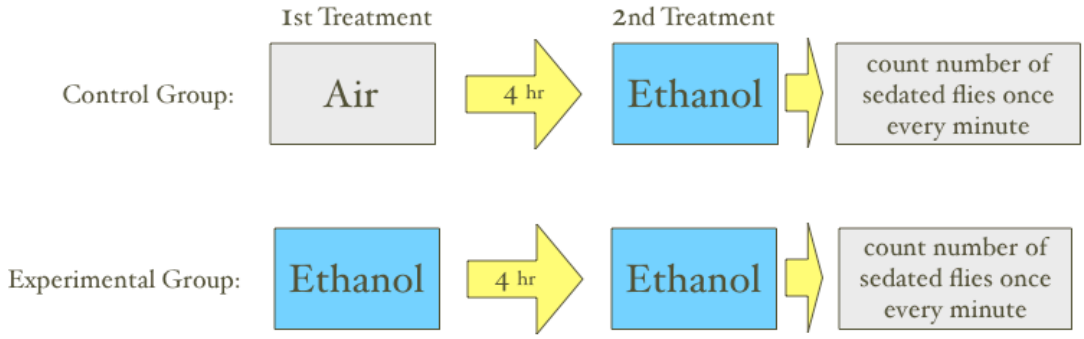


Figure 8. Tolerance protocol.

For the first treatment, half the flies are sedated with ethanol, and half the flies are exposed only to air. For the second treatment, all flies are sedated with ethanol. Recovery times are noted for the second treatment.

I observed that the recovery curve varied from experiment to experiment. Thus, when determining if there was a statistical difference between control and experimental groups, single point measurements, such as t1/2 seemed to be arbitrary and unreliable measures of the recovery of the population. Therefore, I chose to examine the entire recovery curve when trying to evaluate whether a population exhibited tolerance. This required the use of

a statistical test that evaluated the significant difference of entire curves. The consensus of several statisticians was that log rank test of survival analysis was the appropriate statistical measure in this case. This measure takes into account the entire curve, and also accounts for loss of animals during the experiment.

Drosophila can acquire tolerance after a single exposure to ethanol.

Several lines of wild-type flies were tested for tolerance in the tolerance protocol given above. Canton S and Oregon R are wild-type strains common in laboratories. w^{1118} is also a common lab strain that is carries a mutation interfering with an early step in the production of eye pigment. P3 flies are the line of transgenic flies used in previous experiments and have w^{1118} genetic background. All these strains of flies were able to acquire tolerance (fig. 9).

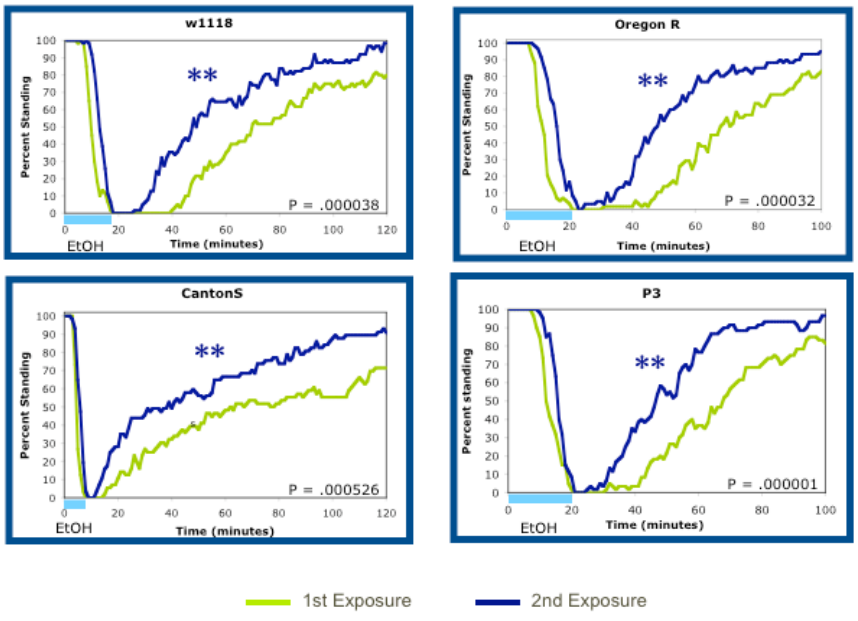


Figure 9. Ethanol sedation and recovery curves for several wild-type stocks of flies. Results shown are 4 hours after first treatment. For all stocks tested, flies remained sedated for a significantly shorter period of time during their second exposure to ethanol. Significance was calculated using log rank survival analysis.

Tolerance persists

To determine how long tolerance persists, the tolerance protocol was modified slightly. A large number of matched Canton S flies were divided into two groups. The flies were placed in large treatment chambers, and one group was exposed to ethanol vapor until sedated while the other group was exposed to a stream of fresh air. The flies were then divided into groups of ten, and at different time points (2 days, 4 days, 7 days, and 14 days) their ethanol tolerance was measured. Specifically, four groups of control and four groups of ethanol-treated flies were treated with ethanol and their recovery from sedation times noted. I observed that tolerance persisted for at least 7 days, but was not apparent after 14 days (fig. 10).

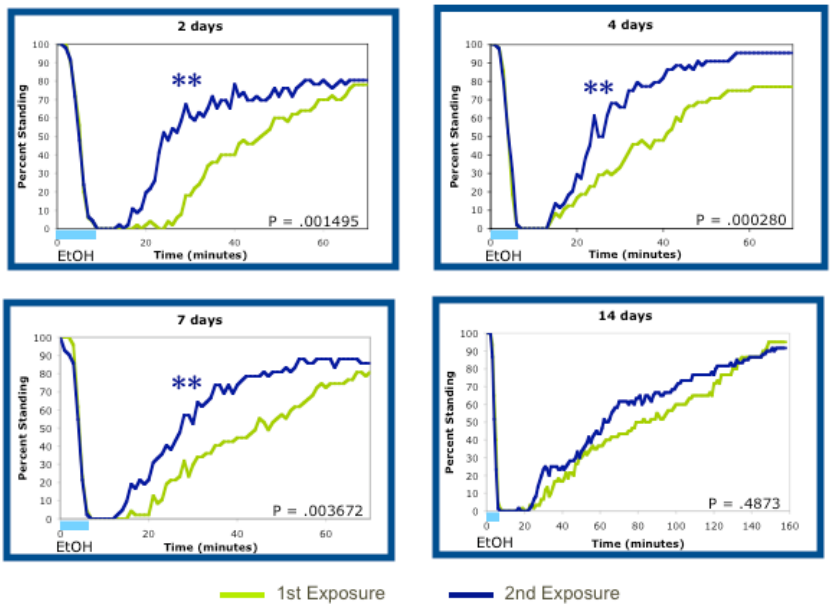


Figure 10. Time course of tolerance.

Canton S flies were tested for tolerance with 2, 4, 7, and 14 days between treatments with ethanol. Tolerance persisted for at least 7 days after a single sedation with ethanol.

slowpoke mutants do not acquire tolerance

Flies mutant for the *slowpoke* gene were tested to determine if they could acquire tolerance as a result of a single ethanol sedation. The slo^4 mutation is a chromosomal inversion with a breakpoint within the gene. It produces no detectable gene product (93). I observed that flies homozygous for the slo^4 mutation were unable to acquire ethanol tolerance in response to a single ethanol sedation (fig. 11).

It is possible that the slo^4 line of flies carry a second unidentified mutation or allelic variation(s) that causes the animals to be unable to acquire tolerance. To eliminate this possibility we tested other mutant lines which carried different genetic lesions that interfere with *slowpoke* expression. To generate a line of *slowpoke* mutants with a different genetic background, slo^4/ slo^4 flies were crossed to the deficiency strain Df(3R)crb 87-5/TM3, which has a large deletion on the third chromosome encompassing the *slowpoke* gene. This chromosome is lethal when homozygous and could not be tested directly. When slo^4/Df(3R)crb 87-5 flies were tested, they were unable to acquire tolerance (fig. 11).

It is also possible that the slo^4 mutation or some other gene on the same chromosome interferes with the manifestation of tolerance in a dominant manner. To rule out this possibility, homozygous slo^4 /slo^4 flies were crossed to the wild-type strain Canton S to generate heterozygous slo^4/+ flies. These flies retained the ability to acquire tolerance (fig. 11), therefore the loss of tolerance is not due to a dominant slo^4 phenotype nor to a another gene carried on the slo^4 chromosome. This also shows that a single copy of the *slowpoke* gene is sufficient for tolerance.

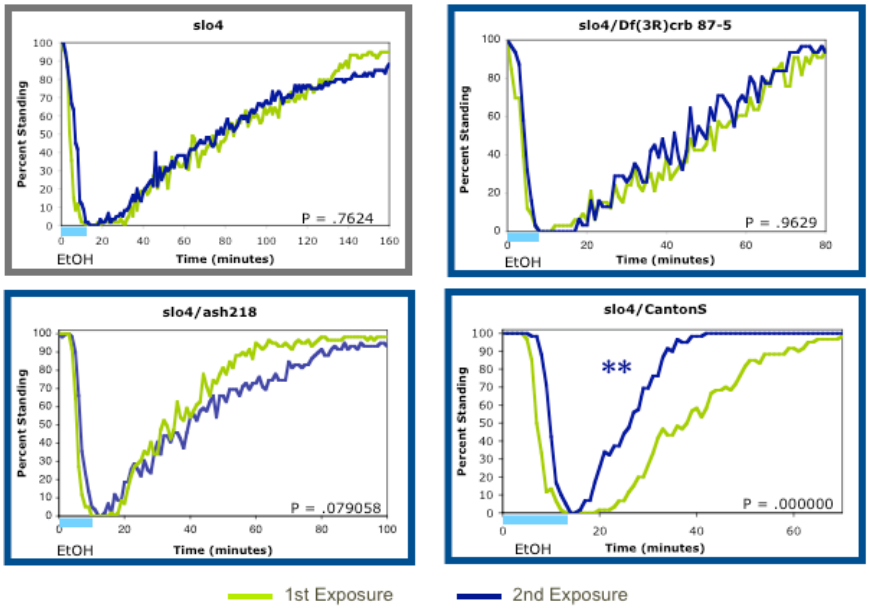


Figure 11. Ethanol sedation and recover curves for several lines of *slowpoke* mutant flies.

Neuronal expression of *slowpoke* is required for tolerance

The *slowpoke* gene has a very complex expression pattern. It is expressed throughout the muscles, nervous system, midgut and trachea of the fly. The transcriptional control region of the *slowpoke* gene is at least 7 kb, and contains five tissue-specific promoters, two neuronal, one for muscle and trachea, and two for the midgut.

Behavioral tolerance to a drug is likely to be caused by changes that affect the signaling properties of the nervous system. To determine if tolerance was associated with the expression of *slowpoke* in the nervous system I generated mutants that lacked *slowpoke* expression only in the nervous system. To do this, slo^4/ slo^4 flies were crossed to $ash2^{18}$/TM6 tb mutants. The $ash2^{18}$ mutation is a large deletion in the third chromosome that removes the neural promoters of *slowpoke*, leaving the muscle and tracheal promoters intact (fig. 12). In addition, it removes the neighboring *ash2* gene. The loss of *ash2* causes the $ash2^{18}$ lesion to be a recessive lethal mutation, but $ash2^{18}/slo^4$ is viable.

These flies produce functional *slowpoke* channels in the muscles, but not the nervous system of the fly (94). These flies were unable to acquire tolerance to ethanol, therefore it is the neural version of *slowpoke* that is critical for tolerance (fig. 11).

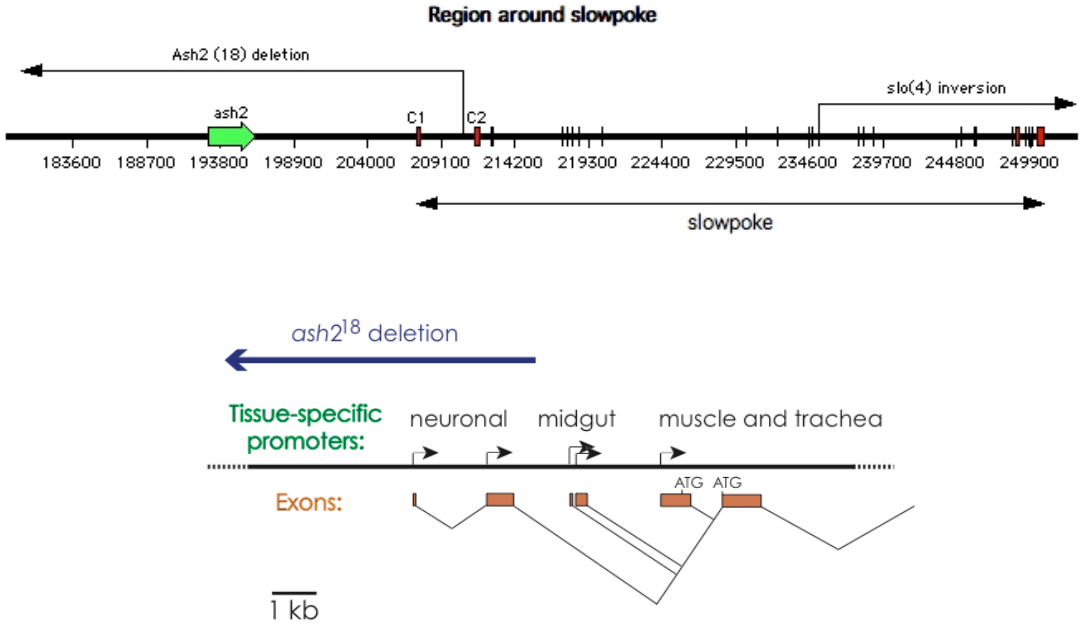


Figure 12. *slo*^{4} and *ash2*^{18} mutations.

Over-expression of *slowpoke* leads to resistance

We have shown that flies increase their expression of *slowpoke* after sedation with ethanol, and that this is coincident with a gain of tolerance. We have also shown that flies lacking *slowpoke* are unable to acquire tolerance to ethanol. The next question we wanted to ask is, if we artificially increased *slowpoke* expression without ethanol exposure, would the flies exposed to ethanol for the first time behave as if they had already gained tolerance. To answer this question we used another line of transgenic flies called B52H. These flies carry a transgene in which a heat shock promoter drives the expression of a *slowpoke* cDNA. In the B52H stock the transgene is carried in a *slo*^{4} background. These

flies do have some basal level of *slowpoke* expression due to leakiness of the heat-shock promoter (85). However when these flies are heat-shocked, their *slowpoke* expression increases greatly. For these tests, matched B52H flies were collected and divided into two groups. In one group, *slowpoke* expression was induced by placing the flies in a 37˚C incubator for 30 minutes, three times over a 24 hour period prior to their first ethanol treatment. The other group of B52H flies remained at room temperature (~21˚C) during this 24 hour period. Twenty-four hours after the first heat-shock, both groups of flies were then placed in the inebriator and exposed to ethanol vapor until all flies were sedated. The ethanol was then withdrawn and the flies were allowed to recover in their chambers. The number of flies sedated was recorded once every minute during the entire treatment and recovery. B52H flies that had *slowpoke* induced (heat-shocked) remained sedated for a shorter period of time than B52H flies which did not have *slowpoke* induced (not heat-shocked). Flies with normal *slowpoke* expression do not display this response to heat-shock (fig. 13).

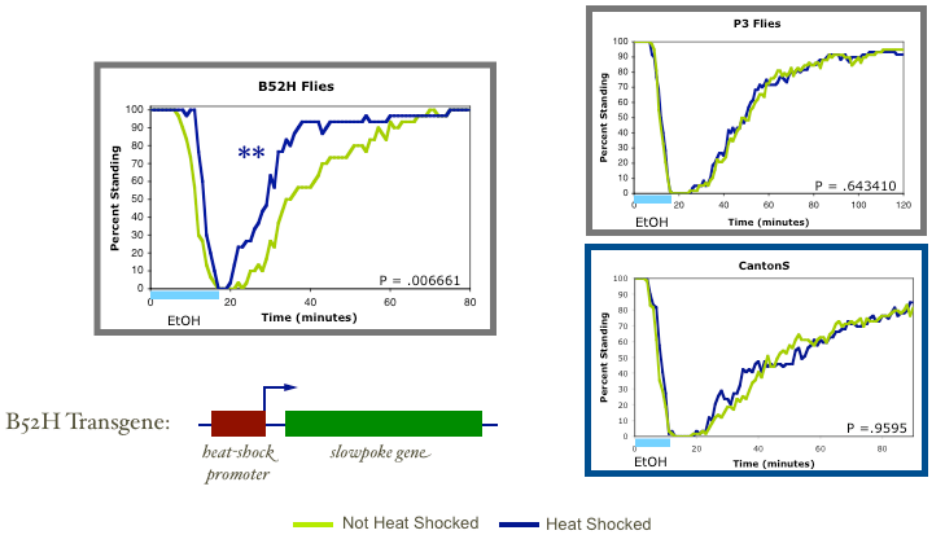


Figure 13. Ethanol sedation and recovery curves for heat-shocked and non-heat-shocked B52H, Canton S and P3 flies.

Heat-shocked B52H flies over-express *slowpoke* and remain sedated for a significantly shorter period of time than non-heat-shocked B52H flies.

Mutants for other ion channels can acquire tolerance.

I have shown that the mRNA abundance of several ion channel genes is increased in response to ethanol sedation. I then wished to determine if mutations of this type which alter neural excitability would also interfere with tolerance. To test this idea, I examined four other ion channel mutations. These were the *cacophony*, a mutation in a voltage-gated calcium channel; *paralytic*, a mutation in a voltage-gated sodium channel; *Shaker*, a mutation in a voltage-gated potassium channel; and SK, a mutation in a small-conductance calcium-activated potassium channel. All these mutants tested were able to acquire tolerance (fig. 14). This indicates that *slowpoke* is unique in its role in the ethanol response.

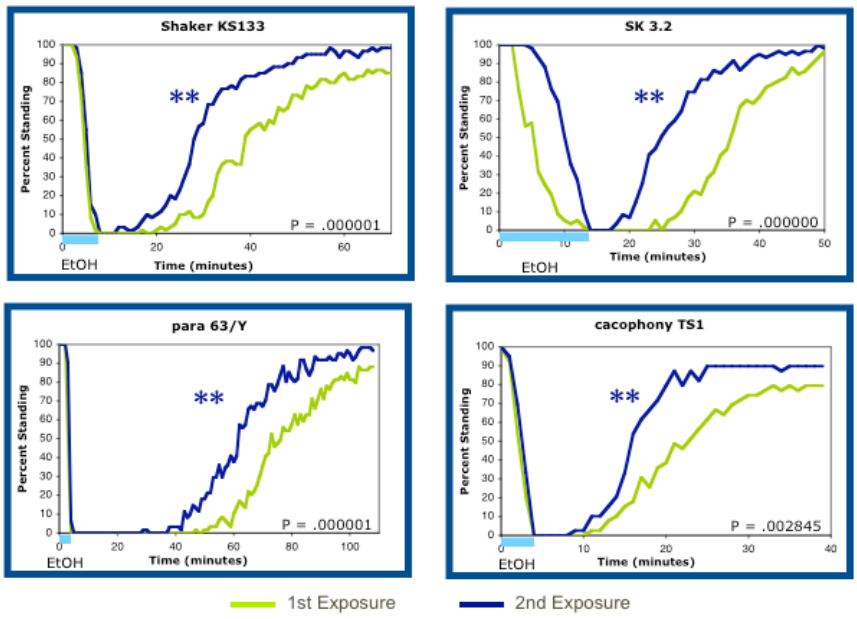


Figure 14. Ethanol sedation and recovery curves for *Shaker*^{KS133}, SK 3.2, *para*^{63}, and *cacophony* TS1 mutant flies.

All strains were able to acquire tolerance to ethanol.

Cross-tolerance

Drosophila have been shown to acquire tolerance to the sedating effects of the volatile anesthetic benzyl alcohol (85). In order to determine if tolerance to benzyl alcohol and ethanol occur via related mechanisms, I tested flies for cross tolerance. To do this, matched wild-type flies were collected and divided into two groups. One group was treated with an sedating dose of benzyl alcohol, the other group was exposed only to air. Twenty four hours later all flies were exposed to ethanol and the number of flies sedated in both groups was recorded once every minute during the entire recovery period. Flies that were previously exposed to benzyl alcohol had acquired tolerance to ethanol despite the fact that they had never been exposed to ethanol before (fig. 15). The reverse experiment was also shown to be true: flies previously exposed to ethanol had tolerance to the effects of benzyl alcohol (fig. 15). Therefore the mechanism of acquisition of tolerance to these two drugs must share common pathways.

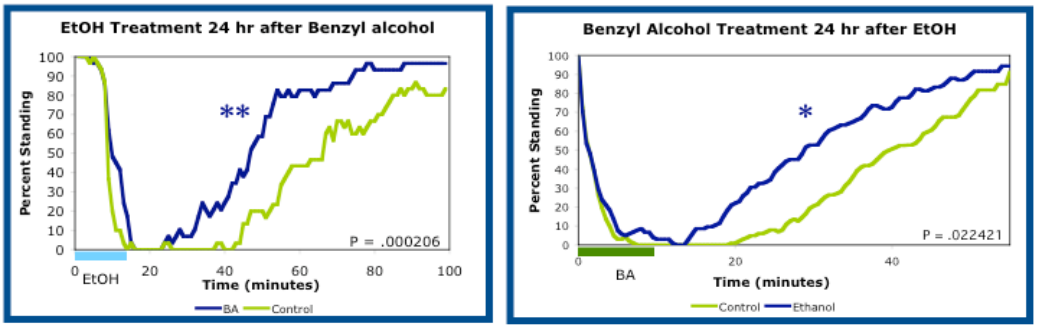


Figure 15. Cross tolerance between ethanol and benzyl alcohol.
Flies were either sedated with benzyl alcohol, then later tested for tolerance to ethanol, or sedated with ethanol and later tested for tolerance to benzyl alcohol. Wild-type flies were able to acquire tolerance in both circumstances.

Conclusions

Several strains of wild-type flies have been shown to acquire tolerance to the sedating effects of ethanol. This is seen as a shorter duration of sedation during the second exposure to ethanol compared to the first exposure. However, flies which are null mutants for the *slowpoke* gene, in several different genetic backgrounds, were unable to acquire tolerance in this manner, as were flies which lacked *slowpoke* expression only in the nervous system of the fly. This demonstrates that the lack of tolerance is not due to another recessive alteration in the *slowpoke* mutant chromosome. While flies which were heterozygous for the *slowpoke* mutation, and wild-type on their other chromosome, retained the ability to acquire tolerance. This demonstrates that there is not a dominant alteration in the *slowpoke* mutant chromosome that could account for the lack of tolerance. Therefore I conclude that it is the *slowpoke* mutation itself that is responsible for the lack of ability to acquire tolerance.

Of course using a mutant organism which lacked a certain gene product during development is not the ideal scenario. The nervous system of the fly may have compensated for the lack of a particular channel by altering the function of other channels (95). However, I was not able to successfully knock out the Slowpoke channel in adult organisms, but I was able to perform the reciprocal experiment on adult flies. Using a line of transgenic flies called B52H, I was able to over-express the *slowpoke* gene at a time of my choosing. If lack of *slowpoke* lead to a lack of ability to acquire tolerance, then it follows that over-expression of *slowpoke* should lead to enhanced tolerance. And this is in fact what occurred. B52H flies contain the *slowpoke* gene under control of a heat shock promoter, when heat shocked these flies will over-express *slowpoke*. B52H flies which were heat-shocked prior to ethanol sedation, remained sedated for a shorter period of time than non-heat-shocked B52H flies. This supports my hypothesis that the *slowpoke* gene itself is involved in the phenomenon of tolerance.

However, there are still many unanswered questions. The initial increase in *slowpoke* expression seen at six hours after ethanol sedation is coincident with a gain of tolerance. This is consistent with the B52H data demonstrating that increasing *slowpoke* levels artificially leads to 'tolerance'. However, I have also demonstrated a decrease in *slowpoke* transcription occurs at twenty-four hours, yet tolerance lasts for at least seven days. I cannot explain this discrepancy, although I do not yet know which, if any, of these changes in *slowpoke* expression, lead to changes in functional channels, nor do I know which other genes are involved on tolerance, and what they are doing at these times.

To further implicate *slowpoke* as a unique gene in ethanol tolerance, I next tested animals mutant for several other ion channel genes, some of which were shown to also be up-regulated in response to ethanol sedation. These other ion channels would also presumably alter the excitability of the nervous system, so testing these other mutants would allow me to determine if any change in excitability by itself is enough to alter the phenomenon of tolerance. The mutants animals tested were $Shaker^{KS133}$, $para^{63}$/Y, $cacophony^{TS1}$, $cacophony^{TS2}$, SK 3.2 and SK 7.2. I could not test mutants for all ion channels as mutant flies are not readily available for all genes.

The *Shaker* gene encodes a rapidly activating and inactivating voltage-gated potassium channel. Flies mutant for this gene were named because they 'shake' under anesthesia. This channel is expressed in the muscle and nervous system of the fly. $Shaker^{KS133}$ is a missense point mutation, loss of the *Shaker* current has been shown to enhance transmitter release (96). Interestingly *Shaker* mutants, but not *para* or *slowpoke* mutants, have been shown to be more sensitive to the volatile anesthetic isoflurane (97). However I have shown that *Shaker* mutants are able to acquire tolerance to ethanol.

The channel encoded by the *paralytic* gene is the predominant voltage-gated sodium channel in the fly. It is expressed widely throughout the central and peripheral nervous system, but not the musculature. Null mutations of this gene are lethal, therefore

temperature sensitive mutants were used. The *para*^{63} mutation is caused by a point mutation and mutant animals show temperature sensitive paralysis and reduced viability (98). Male flies were used in these studies because this gene is on the X chromosome. These flies had a single defective copy of the *paralytic* gene, yet were still able to acquire tolerance to the sedating effects of ethanol.

The *cacophony* mutant was first identified by defects in its mating song. The mutant gene encodes a voltage-gated calcium channel. Unlike vertebrates, in insect skeletal muscle, calcium currents are solely responsible for generation of action potentials, rather than sodium currents. In *Drosophila* there are four genes encoding the α1 subunit of voltage-gated calcium channels which are homologous to the mammalian T-type/α1G, N-type/α1A (Dmca1A), and L-type/α1D (Dmca1D), and two C. elegans (Dmα1U) voltage-gated calcium channels (99). The gene defective in *cacophony* mutants is homologous to the L-type voltage-gated calcium channel from mammals. Null mutations are lethal while partial loss of function mutations cause temperature sensitive convulsions, disrupt synaptic transmission, vision, and courtship (100, 101).Two different temperature sensitive *cacophony* mutants were tested, and both were able to acquire tolerance to the sedating effects of ethanol.

The SK channel is a small conductance calcium-activated potassium channel that has only recently been identified in *Drosophila* (102). The SK 3.2 and 7.2 mutations were generated by transposon insertion in the lab of J.P. Adelman, although a phenotype for the mutant animals has not yet been determined. In mammals, SK channels are gated solely by intracellular calcium ions, and are insensitive to voltage. When activated these channels allow the efflux of potassium ions. These channels are responsible for the slow afterhyperpolarizations seen after an action potential. Their activation has been shown to inhibit cell firing and limits the firing frequency of bursting action potentials (103). Two different *Drosophila* SK mutants were tested, and both were able to acquire tolerance to the sedating effects of ethanol.

Despite the fact that several other ion channel genes were shown to be up-regulated in response to ethanol sedation, other ion channel mutant animals retained the ability to acquire tolerance. Therefore the *slowpoke* channel plays a unique role in the phenomenon of tolerance.

Finally, I have shown that rapid tolerance to the sedating effects of ethanol and benzyl alcohol occur via related mechanisms. Flies sedated with ethanol show a tolerance to benzyl alcohol, and flies sedated with benzyl alcohol show a tolerance to ethanol. Benzyl alcohol is a volatile anesthetic, and other drugs in this class have been shown to have effects upon the nervous system very similar to those of ethanol. Volatile anesthetics, such as halothane and isoflurane, have been shown to potentiate $GABA_A$ (104), glycine (19), nnAChR (105), and 5-HT_3 receptors (106), as well as inhibit NMDA receptors (107) and voltage-gated calcium channels (108). Benzyl alcohol has also been shown to up-regulate neural *slowpoke* in *Drosophila* (85). Therefore it is not surprising that a drug in this class shows cross-tolerance to ethanol.

However these drugs do not have identical effects upon the nervous system of the fly. We have seen differences in the behavior of flies following exposure to these drugs. Both drugs will cause a brief hyperexcitable phase prior to sedation, however other behavior involving sedation are different. Flies sedated with benzyl alcohol are completely immobile, while flies sedated with ethanol usually twitch. Flies sedated with benzyl alcohol seem to recover faster and more completely than flies sedated with ethanol. Finally, flies recovered from sedation with ethanol appear to have a ‘hang-over’. They do not very active, they do not respond to stimuli, and they do not climb the sides of a vial, a typical behavior of flies. This hang-over lasts for weeks after a single sedating dose of ethanol. The same is not seen in benzyl alcohol sedated flies, in fact benzyl alcohol sedated flies seem more active than non-treated flies. Therefore the neurological response of flies to these two drugs, though similar, is not identical.

CHAPTER 4 – ETHANOL METABOLISM

A hypothesis that accounts for the results presented in Chapters 2 and 3 is that the modulation of *slowpoke* expression is an adaptive response of the *Drosophila* nervous system to compensate for at least some of the intoxicating effects of ethanol, and that this change in *slowpoke* expression is at least partially responsible for rapid tolerance to ethanol. Support for this hypothesis is found in our observation that stocks carrying the slo^4 null allele are incapable of acquiring tolerance in response to a single ethanol sedation. Because *slowpoke* encodes a potassium channel it is most likely that this phenotype arose because of the mutation in the *slowpoke* gene, and that it occurs because the mutation alters the electrical properties of the nervous system. However, it is formally possible that *slowpoke* mutants have not only altered signaling properties but also different ethanol pharmacokinetics; that is, that the inability to acquire tolerance may be due, at least in part, to a difference n the metabolism of ethanol between wild-type and *slowpoke* mutant animals. It is also possible that wild-type flies acquire tolerance, not because of adaptations to the nervous system, but because of changes to their metabolism of ethanol.

In flies, like humans, alcohol dehydrogenase (Adh) accounts for the majority of initial ethanol metabolism. In flies, Adh metabolizes ninety percent of ethanol to acetaldehyde (76). This acetaldehyde is then converted to acetic acid, though unlike humans, this is done primarily by Adh, and only partially by aldehyde dehydrogenase (109, 110). The acetic acid is then diverted to the tricarboxylic acid (TCA) cycle for synthesis of amino acids, sugars, and fatty acids (111, 112).

Flies have a single gene that encodes Adh (113). The Adh locus has two tandem promoters (114). The proximal promoter is utilized from mid-embryogenesis through mid-third larval instar. Transcription from this promoter then diminishes and remains low

throughout adulthood. The distal promoter is transcribed at a low rate throughout development and at a high rate during adulthood (115). Transcription from the proximal (larval) promoter, but not the distal (adult) promoter is up-regulated up to five-fold in response to a diet that includes 2.5% ethanol (116, 117). Therefore an up-regulation of Adh is unlikely to account for the tolerance I have observed.

There could, however, be other changes in metabolism that could alter the clearance rate of ethanol and thus could pharmacokinetically contribute to rapid tolerance, such as an alteration in the respiration rate of the flies, or an alteration in other enzymes related to ethanol degradation. It has also been shown that *Drosophila* which carry null mutations in the Adh gene remain sedated for longer periods of time than wild-type animals(118). Therefore I monitored the metabolism of ethanol throughout the different stages of tolerance for both wild-type and *slowpoke* mutant flies to determine if it could account for either the tolerance observed in wild-type animals or the inability to acquire tolerance observed in animals that carry mutations in the *slowpoke* gene. To do so, I measured the absolute amount of ethanol in the flies at different time points throughout the recovery phase.

Metabolism is not altered

Age and sex matched Canton S and slo^4 flies were collected and divided into two groups each. One group of Canton S and one group of slo^4 flies were put in the inebriator and sedated with an air-stream saturated with ethanol. The second group of CantonS and slo^4 flies were also placed in the inebriator, but were exposed only to fresh air. After all the ethanol-exposed flies were sedated, they were switched to fresh air and allowed to recover within their test chambers. The control flies also remained in their treatment chambers during this recovery period. Once all ethanol-sedated flies had recovered, all flies were transferred to food vials. Four hours after the initial ethanol treatment, all flies, were put back in the inebriator and sedated with ethanol. However, unlike all previous

experiments, they were not allowed to recover in their chambers. All flies, while still sedated, were immediately removed from their chambers and carefully put into food vials, five flies per vial. Three vials from each of the four groups were selected every 25 minutes and the flies were transferred to gas chromatography vials. These vials were immediately placed in a -20˚C freezer to stop all metabolic processes (fig. 16).

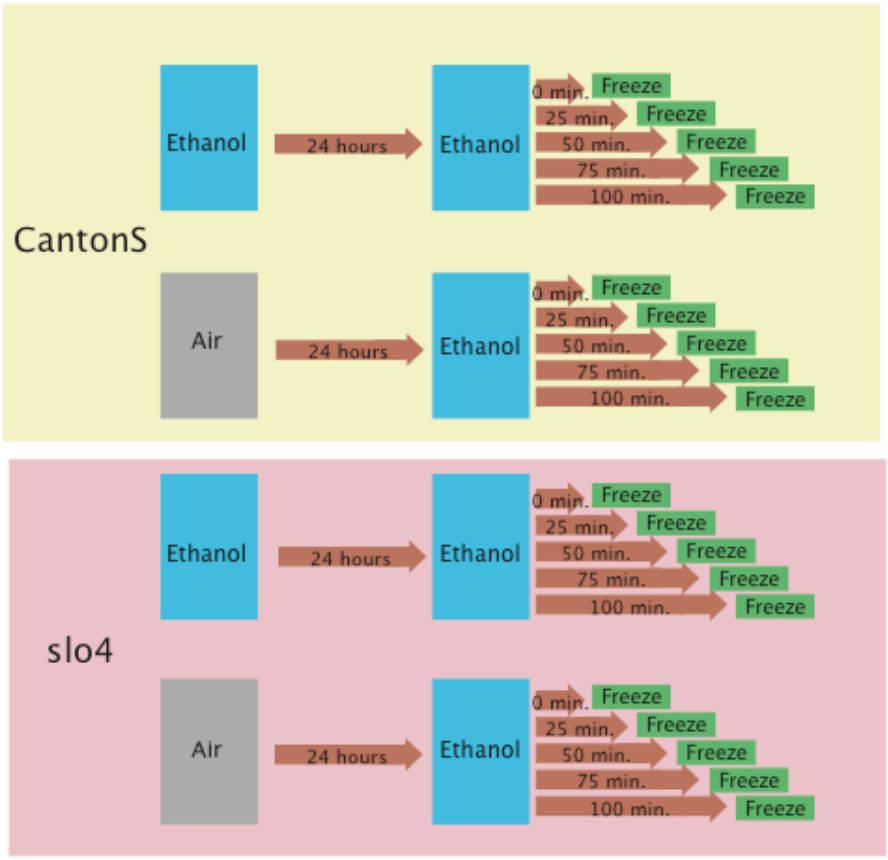


Figure 16. Protocol for measuring metabolism of ethanol.

Four groups of flies were examined: two groups of slo4 mutants, naïve and ethanol-treated, and two groups of wild-type Canton S, naïve and ethanol-treated. All groups were sedated with ethanol during the second treatment. Absolute ethanol levels were quantified at various time points after this second treatment.

The weight of the flies in each vial was also noted before each vial was frozen by weighing the vial before and after addition of flies.

After all flies had been frozen, the vials of flies were placed in a gas chromatograph and ethanol levels were quantified against an ethanol standard curve (fig.17). The results reveal a very similar rate of ethanol metabolism for all groups of flies.

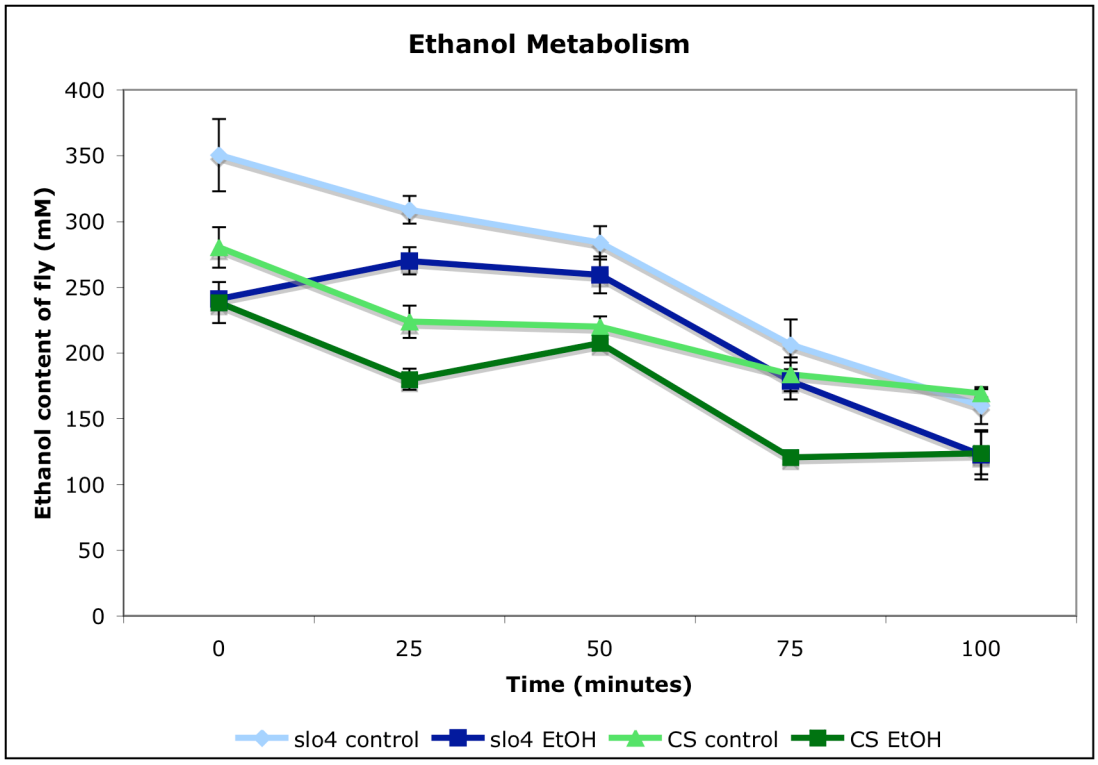


Figure 17. Ethanol concentrations in four groups of flies after their second treatment.

These results also gives an ethanol content of about 240 mM in wild-type flies immediately after sedation. A second method was also used to quantify absolute ethanol levels in wild-type flies immediately after ethanol sedation. In this method, flies were sedated with ethanol, then immediately homogenized in a buffer. This lysate was then

assayed for ethanol content against an ethanol standard curve using an enzymatic assay. This method gave a value of 235 mM.

Conclusions

I have shown that the rate of elimination of ethanol following ethanol sedation was not significantly different in wild-type and *slo*^{4} null mutant flies, when comparing their first exposure to their second exposure, or comparing mutant to wild-type directly. Slowpoke mutants do have slightly more ethanol in their system, but it is eliminated at a similar rate as wild-type flies. Therefore the tolerance, or lack of tolerance, observed previously was most likely due to pharmacodynamic changes, and not pharmacokinetic changes.

I have also calculated the ethanol content of a fly after sedation with ethanol. Results from the gas chromatograph gave 240 mM, while an enzymatic assay gave a very similar value of 235 mM. The Heberlein lab has also done a similar experiment, using an enzymatic assay, and determined that ethanol metabolism does not change in wild-type flies with repeated exposure to ethanol. They have also reported an ethanol concentration of 32 mM in flies immediately after ethanol sedation (80). However their sedation protocol was slightly different from mine. They exposed flies to ethanol vapor for exactly 20 minutes. They chose this time as it was the mean elution time from their Inebriometer. In the Inebriometer, flies are placed at the top of a series of baffles inside a column. They are then exposed to ethanol vapor, as the flies lose postural control, they fall down the tube. Flies which emerge at the bottom of the tube are deemed sedated. However, the behavior resulting from ethanol exposure is not limited to sedation. The initial stages of ethanol intoxication in flies include hyperexcitability and incoordination, which could also cause the flies to fall through the baffles and emerge from the inebriator before being completely sedated. Also, 20 minutes was the 'mean' elution time, there were certainly flies which were not sedated at this point. In fact I have seen enormous fly to fly variability in time to sedation. Finally, when making their calculations, they assumed a

volume of 2 μl for a fly (78). I have taken a variety of measurements and have calculated that the water content of a typical wild-type fly is actually 0.99 μl. Therefore I suspect my value of 240 mM is more likely to be accurate.

In a human 240 mM would correspond to a blood alcohol level of about 1.2% (table 1). This would, of course, be fatal. However flies are not humans. Their physiology is substantially different and it is not surprising that they would have a different ability to withstand the toxic effects of ethanol.

Blood Alcohol Concentration	Molarity	Behavioral effects
Less than 0.05 %	Less than 11 mM	Increased sociability and euphoria
0.05 % – 0.10 %	11 mM – 22 mM	Disturbances in gait, concentration and reaction time
0.01 % - 0.15%	20 mM – 30 mM	Ataxia, impaired mental and motor skills, impaired short term memory, slurred speech
0.20 %	43 mM	No response to sensory stimuli
0.25 %	54 mM	Coma
More than 0.50%	More than 100 mM	Death

Table 1. Human blood alcohol concentrations and the subsequent effects on behavior (119).

CHAPTER 5 – MATERIALS AND 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 7 days later. For all experiments, unless otherwise noted, female flies were used.

Wild-type stocks were Canton S; Oregon R; and w^{1118} (tested because the B52H, P3, and P6 transgenes are in a w^{1118} background).

Genotypes of mutant stocks were *slo*^{4}; *ash2*^{18}/TM6 tb; Df(3R) crb87-5,st[1] e[1]/TM3; *Sh*^{KS133}; *para*^{63}/Y; *cac*^{TS1}; *cac*^{TS2}; SK3.2; and SK7.2.

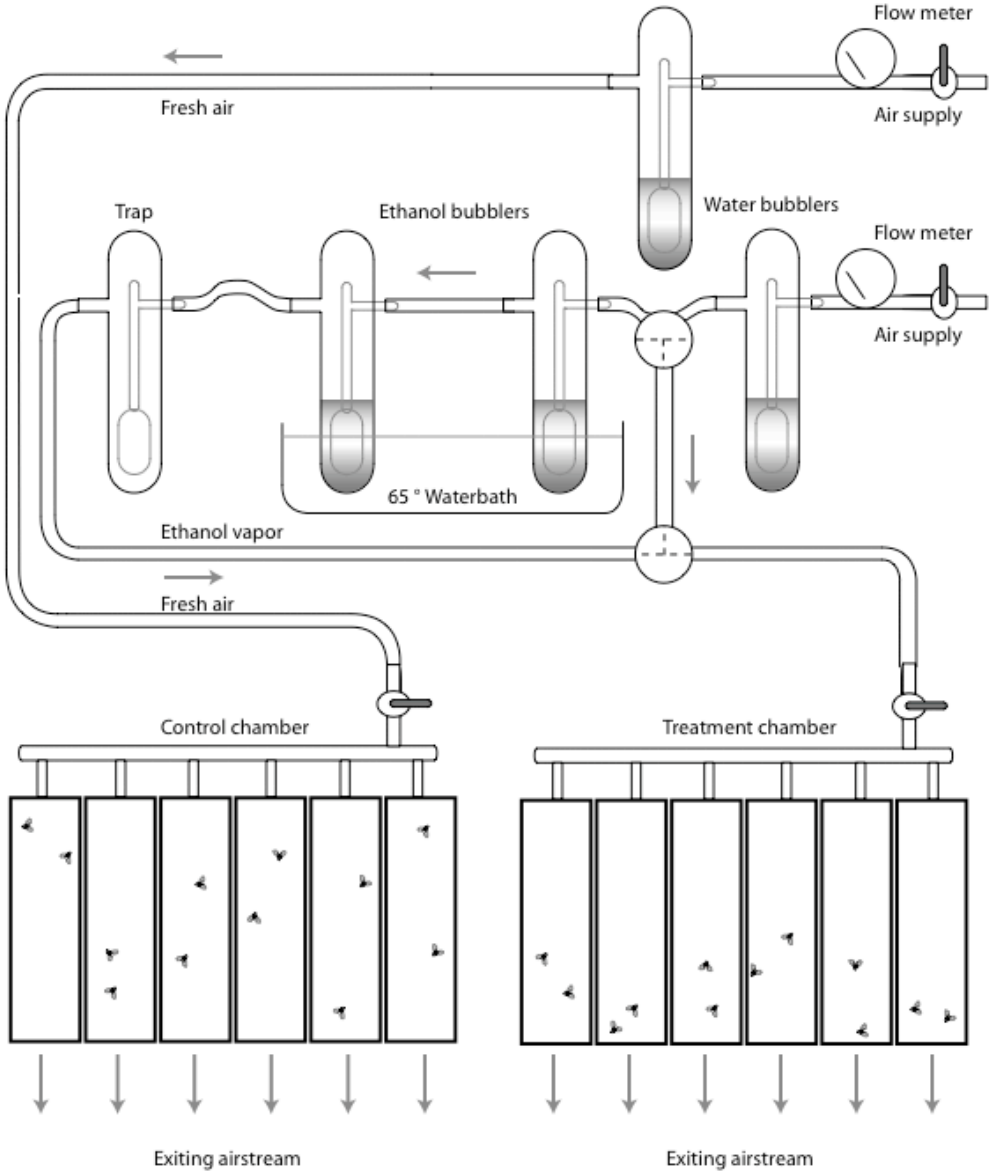
Transgenic flies used were B52H, P3, and P6. The genotype of the B52H transgenic stock is w^{1118}, B52H, *slo*^{4}. In the B52H transgene, an inducible hsp70 promoter drives expression of a *slowpoke* cDNA whose splice pattern is found in the nervous systems and muscles(120). P3 and P6 transgenes are in a w^{1118} background. P3 contains the neuronal transcriptional control region of *slowpoke* while P6 contains the muscle/tracheal cell specific region of the *slowpoke* transcriptional control region. Both P3 and P6 drive expression of a *ß-galactosidase* gene which is terminated by an SV40 polyA adenylation signal (74).

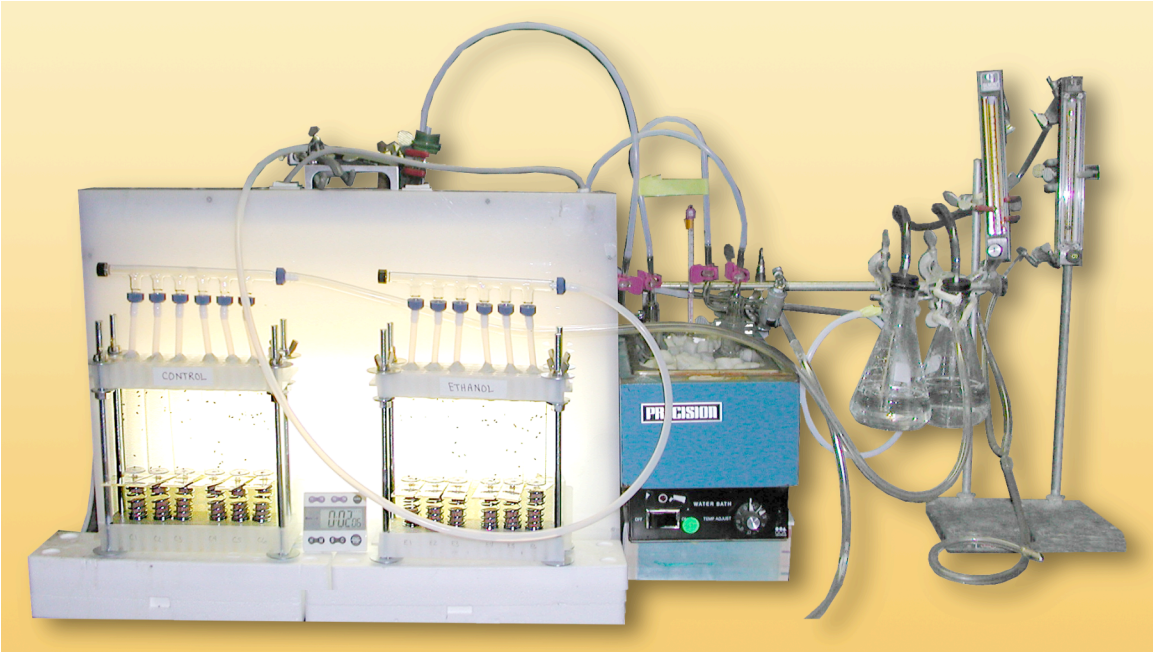
Ethanol sedation/Inebriator

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/min (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 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 was constructed just like the water bubblers.

A treatment chamber consisted of two microfuge tube racks clamping together 6 standard plastic Drosophila 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.





Ethanol tolerance

Sex and age-matched flies were assembled into 12 groups of 10 flies each. This includes six control and six experimental groups. 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 (4 or 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 animals have recovered. Tolerance was quantified during this second treatment by counting the number of flies recovered from sedation in each vial once every minute from the time the ethanol was first applied until the flies had recovered (sometimes a few flies take many, many hours to wake up, and sometimes a few flies die). The results were graphed as the percent of flies recovered from sedation over time for both the control and experimental groups.

Statistics

The Log Rank test for equality of survival was used to determine the significant difference between recovery curves because survival analysis is best suited for data in which one is measuring the time to a specific event (121). In all of the tolerance assays I measure the time that it takes for each fly to recover from sedation. The statistic evaluates if entire recovery curves are statistically different (as opposed to individual data points comprised by the curve).

Benzyl alcohol - ethanol cross tolerance

To determine if sedation with benzyl alcohol led to tolerance to ethanol, sex and age-matched wild-type flies were first exposed to benzyl alcohol. Exposure was performed by coating three 30 ml glass vials with 200 μl of a solution of 0.3% benzyl alcohol in acetone. These experiments included three acetone control vials. The vials were continuously rotated for 45 minutes at 22˚C to evaporate the acetone, leaving a thin coat of evenly distributed benzyl alcohol. Twenty-five flies were placed in each vial and exposed to the benzyl alcohol until sedation (10 to 15 minutes). Twenty four hours later,

both control and benzyl alcohol treated flies were sedated with ethanol and recovery times noted as described above in the tolerance protocol.

To determine if sedation with ethanol led to tolerance to benzyl alcohol, sex and age-matched wild-type flies were first sedated with ethanol as described above. Twenty-four hours later, treated and control flies were simultaneously exposed to benzyl alcohol as described above. Snapshots were taken every 20 seconds during the course of exposure and recovery and stored as a stop-motion movie. Recovery from anesthesia was scored as the return of geotactic behavior. Flies on the walls of the tube were scored as recovered. Values for recovered flies were then plotted as a percentage of the population in each tube (average of three tubes) against time at 20-second intervals.

Quantitative real-time PCR

Sex and age matched w^{1118} flies were placed in the Inebriator: four vials of 7-8 flies each were exposed to ethanol until sedated, while four vials of 7-8 flies each were exposed to air only. After all ethanol-exposed flies were sedated they were switched to fresh air. All flies, control and ethanol, were left in their chambers until all ethanol-sedated flies had recovered. All flies were then transferred to food vials. Total RNA was extracted six or twenty-four hours following the start of treatment using the single-step RNA isolation from cultured cells or tissue protocol (122). The RNA was treated with RNase free DNase I (Ambion Inc. Austin, TX) to remove all DNA contamination. RNA was quantified using the RiboGreen® RNA Quantitation Kit (Part number R11490, Molecular Probes, Inc., Eugene OR) according to manufacturer instructions.

First-strand cDNA was synthesized from 100 ng of total RNA, primed with 200 mM each of gene specific primers for the gene being tested with Superscript II reverse transcriptase (Life Technologies, Gaithersburg, MD). The cDNA was amplified by real-time PCR in an ABI Prism 7700 Sequence Detection System (Applied Biosystems, Foster City, CA)

in the presence of gene specific dual-labeled single-stranded probes. PCR was performed using the TaqMan probes and the TaqMan Universal PCR Master mix (Applied Biosystems). Each PCR was performed in triplicate and the yields thereof expressed as an average. mRNA abundance was quantified using the standard curve method. Significance was calculated using the Student's t-Test.

The primers used were:

cyclophilin I
Upper: 5' – ACCAACCACAACGGCACTG – 3'
Lower: 5' – TGCTTCAGCTCGAAGTTCTCATC – 3'
Probe: 5' – (FAM) – CGGCAAGTCCATCTACGGCAACAAGTT – (TAMRA) – 3'

slowpoke exon C1
Upper: 5' – AAACAAAGCTAAATAAGTTGTGAAAGGA – 3'
Lower: 5' – GATAGTTGTTCGTTCTTTTGAATTTGA – 3'
Probe: 5' – (FAM) – AGAAACTGCGCTTAGTCACACTGCTCATGT – (TAMRA) – 3'

slowpoke exon C2
Upper: 5' – GCTATTTATAATAGACGGGCCAAGTT – 3'
Lower: 5' – GGAAATCCGAAAGATACGAATGAT – 3'
Probe: 5' – (FAM) – CTCAGCCTCACAATGCGAAACGGA – (TAMRA) – 3'

Shaker
Upper: 5' – GCGGATTAAGGTTTGAGACACAA – 3'
Lower: 5' – GTACCGTAATCTCCGAGCTGGAT – 3'
Probe: 5' – (FAM) – CGTTAAATCAATTCCCGGACACGCTG – (TAMRA) – 3'

paralytic

Upper: 5’ – GGTGCTGCGAGCGCTTAA – 3’

Lower: 3’ – GGATAATCACATCGCGCAGAT – 3’

Probe: 5’ – (FAM) – ACCGTAGCCATTGTGCCAGGCTTGA – (TAMRA) – 3’

cacophony

Upper: 5’ – TAGTGAAGGAGGGCGAATCAGA – 5’

Lower: 5’ – GCTTGTGGTGTTATTGCATACGAA – 3’

Probe: 5’ – (FAM) – TTGCAACACGGACAACATCCTGGAA – (TAMRA) – 3’

seizure

Upper: 5’ – TGGTGATGTACACGGCCATT – 3’

Lower: 5’ – ATGACAATTGGATCGGAGTTGAT – 3’

Probe: 5’ – (FAM) – TCACGCCGTACGTGGCTGCC – (TAMRA) – 3’

shal

Upper: 5’ – CCGTGTCTTCCGCATATTCA – 3’

Lower: 5’ – ATGACGGTGGCAAAGATGATAA – 3’

Probe: 5’ – (FAM) – TTCGGATCCTCGGCTA – (TAMRA) – 3’

shaw

Upper: 5’ – GTCCTGGGCATCGTGATCTT – 3’

Lower: 5’ – TAGCCGACGGTGGTCATTGT – 3’

Probe: 5’ – (FAM) – CGCGGAGCGCAATCCAGCC – (TAMRA) – 3’

ß-gal assay and protein assay

For the *ß-galactosidase* and protein assays, P3 or P6 flies were divided into groups of ten flies each. Whole fly lysate was made from a group of 10 flies by homogenizing them in 1 ml of Assay Buffer (50 mM KPO_4, 1 mM $MgCl_2$), then centrifuging to remove debris.

ß-galactosidase levels in the lysate were measured using the BIO-RAD FluorAce ß-Galactosidase Assay Kit according to manufacturer's instructions (Part number 170-3150, BIO-RAD Laboratories, Hercules, CA). The protein assay was performed on the lysate using BIO-RAD dye concentrate according to manufacturer's instructions (Part number 500-0006, BIO-RAD Laboratories, Hercules, CA). For each group of ten flies, *ß-galactosidase* levels were normalized against protein levels. Each data point is the average of three groups of ten flies each. Wild-type flies have a small level of endogenous beta-galactosidase activity that was accounted for by subtraction. Significance was calculated using the Student's t-Test.

Gas Chromatography

Age and sex matched Canton S and *slo*^{4} flies were collected and divided into two groups each. One group of Canton S and one group of *slo*^{4} flies were put in the inebriator and sedated with an air-stream saturated with ethanol. The second group of CantonS and *slo*^{4} flies were also placed in the inebriator, but were exposed only to fresh air. After all the ethanol-exposed flies were sedated, they were switched to fresh air and allowed to recover within their test chambers. The control flies also remained in their treatment chambers during this recovery period. Once all ethanol-sedated flies had recovered, all flies were transferred to food vials. Four hours after the initial ethanol treatment, all flies, were put back in the inebriator and sedated with ethanol. All flies, while still sedated, were immediately removed from their chambers and carefully put into food vials, five flies per vial. Three vials from each of the four groups were selected every 25 minutes and the flies were transferred to gas chromatography vials. These vials were immediately placed in a -20°C freezer. The weight of the flies in each vial was also noted before each vial was frozen by weighing the vial before and after addition of flies. After all flies had been frozen, the vials of flies were placed in a Varian CP-3800 gas chromatograph and ethanol levels were quantified against an ethanol standard curve. Each value was normalized against the weight of the flies.

Enzymatic ethanol assay

Sex and age matched flies were divided into groups of 10 flies each and sedated with ethanol in the Inebriator. Twenty-four hours after ethanol sedation, each group of flies was homogenized in 1 mL of 50 mM Tris, pH 7.5 and centrifuged to remove debris. Ethanol was quantified by mixing 5 μl of lysate with 1000 μl of Alcohol Reagent (Sigma part number 333-100), incubating for 10 minutes at room temperature, and measuring absorbance at 340 nm. Quantities were calculated using an ethanol standard curve. A protein assay was also performed on the lysate using BIO-RAD Dye Concentrate according to manufacturer's instructions (BIO-RAD Laboratories, Hercules, CA). Ethanol values were normalized against protein levels.

CHAPTER 6 – DISCUSSION

Ethanol has been shown to interact with several ion channels, altering their function in such a way as to lead to a decreased excitability of the nervous system. I have now shown that transcription of the *slowpoke* gene is altered after exposure to ethanol, and that this is coincident with a gain of tolerance. A simple and satisfying hypothesis to explain the role of *slowpoke* in tolerance would be that *slowpoke* plays an excitatory role in the nervous system, and it is up-regulated following ethanol sedation as a homeostatic mechanism to counteract the depressive effects of ethanol, and it is this up-regulation of *slowpoke* itself that is responsible for tolerance.

Consistent with this simple hypothesis is the fact that *slowpoke* is up-regulated six hours after ethanol sedation in wild-type flies, and this is coincident with acquisition of tolerance. If my hypothesis were true, then this up-regulation of *slowpoke* would lead to an increase in the excitability of the nervous system, and this increased excitability would enable the nervous system to better withstand the depressive effects of ethanol upon the next exposure. Also consistent with my hypothesis is the fact that flies which are mutant for the *slowpoke* gene are unable to acquire tolerance and flies which artificially over-express *slowpoke* display 'tolerance' despite the fact that they were never exposed to ethanol. If the up-regulation of *slowpoke* was the reason flies became tolerant, then flies which lack *slowpoke* altogether should, of course, not be able to up-regulate slowpoke and therefore gain tolerance, while flies which already had their *slowpoke* up-regulated, would already have the excitability of their nervous system increased, therefore they would already be tolerant. Also consistent with my hypothesis that *slowpoke* plays an excitatory role is the casual observation that *slowpoke* mutant flies appear less active than wild-type flies and flies which over-express *slowpoke* are more active than wild-type animals.

However there are some inconsistencies with this hypothesis. For instance, s*lowpoke* is down-regulated twenty-four hours after ethanol sedation, a time when tolerance is still in effect. If *slowpoke* plays an excitatory role, and this excitation is responsible for tolerance, then flies with less *slowpoke* should be sensitized to ethanol, not tolerant, because theoretically their nervous system would be depressed at this point. There are also several lines of evidence that point to *slowpoke* playing an inhibitory role, rather than an excitatory role in the nervous system of the fly. The acute effects of ethanol on the nervous system generally include a potentiation of inhibitory channels and an inhibition of excitatory channels (9), and it has been shown in many preparations that *slowpoke* channels are potentiated by acute exposure to ethanol (67-69), which would be typical of inhibitory channels. The *slowpoke* channel also conducts potassium ions, which typically decreases the excitability of a cell. Indeed *slowpoke* has been demonstrated to be inhibitory in many (86-88), though not all(89, 90), preparations in the fly.

An alternate hypothesis to explain my results is that *slowpoke* is playing an inhibitory role in the nervous system. If this were the case then the down-regulation seen at twenty-four hours could be a homeostatic response of the nervous system, an attempt to increase excitability, and counteract the depressive effects of ethanol. If *slowpoke* does play an inhibitory role in the nervous system of the fly, then the lack of tolerance in *slowpoke* mutants might be because they were already 'maximally tolerant'. In other words, perhaps the nervous system of the fly down-regulates this inhibitory channel as a way of increasing its excitability, and it is this increase in excitability that causes tolerance. However, *slowpoke* mutants lack the channel altogether, so these channels could not be down-regulated any further, therefore the nervous system could not increase excitability by this mechanism. If this scenario were true, then comparing wild-type flies to *slowpoke* mutants upon their first exposure to ethanol should reveal that *slowpoke* mutants were more resistant to the sedating effects of ethanol. I have not yet done this experiment, as it would only be valid if the wild-type and mutant animals had the same genetic background. This is because there are certainly other genes involved in sensitivity to

ethanol, and flies with different genetic backgrounds might have slight variations in the sequence of these other genes which could influence their sensitivity to ethanol. However, the *slowpoke* mutants that I have observed appear, if anything, to be more sensitive to ethanol. Mutants typically become sedated faster and recover from sedation much more slowly than wild-type flies. Though again, they are not in the same genetic background as the wild-type flies I have observed so no generalizations should be made.

However, there are also some inconsistencies with the hypothesis that *slowpoke* is playing an inhibitory role in the nervous system. If this were true, then flies which over-express *slowpoke* should have decreased excitability of their nervous system, and therefore display sensitivity to ethanol, rather than the tolerance I have observed. Another observation not explained by this second hypothesis is the up-regulation seen at six hours after ethanol sedation. But it could be explained as an idiopathic response of the nervous system, not be intended to accomplish any particular function, but having unfortunate side-effects, such as a decreased excitability of the nervous system at a time when it is already depressed. However, the initial up-regulation might not necessarily lead to increased *slowpoke* channel activity. In fact it has recently been shown in the rat hypothalamic-neurohypophysial system that the potentiation of *slowpoke* channels observed after acute ethanol exposure actually starts to diminish within 12 minutes of treatment and is almost non existent by 24 hours. It was also shown in this system that at 24 hours, channel density had decreased due to internalization of the channels, although total channel number did not change (73). So despite any changes I have seen in transcription, there could still be many other factors affecting its function, not just localization, but also phosphorylation state and binding of accessory proteins.

Another point to consider when formulating a hypothesis about the role of *slowpoke* is the fact that there may be very well specific neural circuits involved in tolerance. The up- and down-regulations I have observed may not be representative of what is occurring in these circuits, but instead are an average of many alternate changes occurring in different

brain regions, many of which may be irrelevant to the phenomenon of tolerance (but perhaps relevant to other ethanol-induced behaviors). For example, *slowpoke* is expressed widely throughout the fly brain, including visual areas, the antennae, and the mushroom body (93). The mushroom body has been implicated in learning and memory and control of complex behaviors in the fly (123). However it has recently been shown that ablating the mushroom body altogether did not alter sensitivity to the sedating effects of ethanol in fruit flies (81).

Therefore different brain regions certainly play different roles in the ethanol response, and a better understanding of the specific neurons involved in the phenomenon of tolerance is required. This may help answer the question of whether *slowpoke* is playing an excitatory or inhibitory role. For example, if a critical area of expression of *slowpoke* for the phenomenon of tolerance is in inhibitory interneurons, then *slowpoke* could be both inhibitory in the cell specifically, yet excitatory overall. The *slowpoke* channel also undergoes extensive alternative splicing and different splice variants have been shown to have different functional properties. It is possible that different splice variants are expressed in different brain regions, and that these different splice variants might have different responses to ethanol.

Another point to consider is that I have only examined the role of *slowpoke* in a single ethanol-induced behavior, the duration of sedation, as measured by how long it takes a fly to stand up after sedation with an acute dose of ethanol. There are many other behaviors I could have examined, such as the effect of ethanol on locomotion or pain sensation. I also have not examined *slowpoke* expression during chronic use or withdrawal. Therefore any valid explanation of the role of *slowpoke* in the ethanol response will very likely be more complex than I could formulate at this time.

Finally, *slowpoke* is not the only ion channel whose regulation was altered by ethanol. *Shaker*, *paralytic*, *cacophony*, *seizure*, *shaw*, and *shal* ion channels were all up-regulated

following ethanol sedation, and there are very likely many, many other changes occurring during the time tolerance persists. Therefore it would be an colossal oversimplification to assume *slowpoke* is responsible for all aspects of tolerance.

Interestingly, while I was in the midst of conducting this research, research came out, also implicating the *slowpoke* gene as important in the ethanol response. The *slowpoke* gene was identified in a mutant screen of *C. elegans* looking for alterations in sensitivity to ethanol. *C. elegans* which were mutant for the *slowpoke* gene had hyperactive neurotransmission and were resistant to the depressive effects of ethanol on locomotion and egg laying, while *C. elegans* which over-expressed *slowpoke* behaved as if intoxicated (124). At first this seemed contrary to my findings, however they were examining a different aspect of ethanol, sensitivity and resistance rather than tolerance. Although I suspect that *slowpoke* mutant flies, when they are finally tested for sensitivity or resistance to ethanol, might actually show sensitivity. Although this would be contrary to recent finding in *C. elegans*, it would not be very surprising. The function of the *slowpoke* gene can vary greatly depending on alternative splicing, post-translational modifications, and interactions with other genes, so an altered function in an organism with a fundamentally distinct nervous system would be possible. The point to consider is that *slowpoke* has again been implicated as important in the response to ethanol.

Regardless of the specific function of *slowpoke* in the neurons responsible for ethanol sensitivity or tolerance, I am confident that the *slowpoke* gene plays an important role. I suspect that *slowpoke* might indeed be playing an excitatory role in the cells in question and that the changes in expression are part of a complex homeostatic mechanism to restore the appropriate excitability to the nervous system.

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