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Activity-Dependent Regulation of Ion Channel Gene Expression: A Homeostatic Hypothesis for Drug Tolerance

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Activity-Dependent Regulation of Ion Channel Gene Expression:
A Homeostatic Hypothesis for Drug Tolerance

by

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Dedication

To my parents, Pilar and Eduardo.

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Activity-Dependent Regulation of Ion Channel Gene Expression: A Homeostatic Hypothesis for Drug Tolerance

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Conservation of the balance between excitation and inhibition of neural activity is critically important for the proper function of the nervous system. Upon alterations in excitability, the nervous system may thus trigger mechanisms that attempt to restore homeostasis. Many alcohols, anesthetics, and other abused volatile solvents such as ethanol, benzyl alcohol, toluene, trichloroethylene, and chloroform alter neural excitability and trigger homeostatic adaptations that through the modulation of gene expression and signaling between nerve cells act to counteract these alterations. Many of these adaptations may account for the development of tolerance, dependence and addiction to these drugs. Here, I demonstrate that in Drosophila, tolerance to the sedative effects of alcohols and anesthetics, is mediated by an increase in expression of the Ca^{2+} activated K^{+} channel gene, *slowpoke*. A mutation that eliminates *slowpoke* expression prevents tolerance, while expression

from an inducible *slowpoke* transgene mimics tolerance in naive animals. Furthermore, the behavioral and molecular response to volatile solvents can be separated into an initial phase of hyperkinesis that causes a drop in *slowpoke* gene expression and makes animals more sensitive to subsequent sedation by these drugs, and a sedative phase that stimulates *slowpoke* gene expression and induces tolerance. This demonstrates that the changes in expression level of *slowpoke* act as a modulator of drug sensitivity. Because of it's central role as a regulator of electrical activity in nerve terminals this channel gene is a likely contributor to the homeostatic mechanism that resists untoward changes in net cellular excitability and mediates tolerance to sedation. If hyperexcitability is induced, the proposed mechanism alters channel expression to reduce this excitability, whereas if cellular excitability is suppressed, channel gene expression changes to enhance excitability. An electrophysiological test of this hypothesis shows that increased *slowpoke* expression enhances the excitability of a neural pathway in a way that opposes the effects of sedative drugs. This data supports the notion that *slowpoke* mediated tolerance to sedation is part of a homeostatic adaptation that compensates for changes in neural activity caused by drugs and represents a step forward in the understanding of the molecular basis of drug addiction.

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Chapter 1: General Introduction

The Foundation and Maintenance of Electrical Excitability in the Nervous System

Neural excitability is determined by voltage-gated ion channels

The ability of the nervous system to generate carefully coordinated movements, perform complex computational tasks or exercise precise control over physiological functions, relies on an exquisitely controlled balance between excitatory and inhibitory signals within and between neurons.

The intrinsic excitability of a neuron is determined by the combination of ion channels expressed in these cells. Ion channels are membrane spanning proteins that form ion-selective pores that generally act to maintain an asymmetric distribution of ions across the cellular surface membrane. Commonly, the concentration of sodium (Na^+) and calcium (Ca^{2+}) ions is higher in the extra-cellular space while potassium ions (K^+) are more concentrated inside the cell resulting in the electrically polarized state of a resting cell membrane. Additionally, ion channels display a wide variety of biophysical characteristics that allow them to open and close in response to multiple signals such as chemical neurotransmitters or small changes in the voltage potential of the polarized resting state of the cell. The 'gating' properties of channels allow ions to flow across their concentration and electrical gradients (Hille, 1992).

When activated by small changes in voltage potentials, sodium and calcium channels open transiently to allow these ions to flow into the cell causing a depolarization of the cell membrane. The depolarization of the membrane potential in

turn causes potassium channels to become activated and open to allow potassium ions to flow out of the cell, resulting in the repolarization of the cell membrane. The distribution of a series of these voltage-sensing (voltage-gated) channels along the long projections (axons and dendrites) featured in neurons, allows the transient conduction of ions, that depolarize and repolarize cell membranes, to become rapidly propagated through these projections in the from of electrical regenerating pulses called action potentials (Hille, 1992; Kandel et al., 1991).

When action potentials reach the nerve terminals, the depolarization causes a significant rise in the intracellular calcium concentrations resulting from the activation of membrane voltage-gated calcium channels or other intracellular calcium stores. The increase in calcium levels is thought to mediate the exocytosis of chemical neurotransmitters contained in small intracellular vesicles through the fusion of vesicle to the surface membrane of axon terminals. The release of neurotransmitters to the extracellular space between neurons (synapse) in turn, activates another class of ion channels present in neighboring neurons that act as receptors to neurotransmitters. These ion channels are often referred as ligand-gated ion channels and become activated by neurotransmitter binding. Once activated, ligand-gated ion channels allow the flow of certain ions into or out of the cell. The flow of ions may in some cases cause the depolarization of the cell membrane and the generation of new action potentials, or in other cases further polarize the cell membrane resulting in the inhibition of the action potential generation (Kandel et al., 1991).

Although voltage- and ligand-gated ion channels can be divided into a few number of classes based on their ion-selectivity (i.e. sodium, calcium, potassium

amongst others) or on their ligand recognition, each class is actually composed of a large family of channels with distinct biophysical characteristics. For example, different channels exhibit different gating kinetics (fast or slow opening/closing), have different ion conductance (large or small ion flow) and have distinct ion-selectivity stringency (some allow more that one ion type through the pore). It is the combination of different ion channels present in neurons and other cells what generates the large diversity and uniqueness of electrical signals and firing patterns observed in the different cell types (Hille, 1992).

In recent years it has become evident that of all voltage-gated ion channels families, the potassium channel family is the largest and most diverse, giving rise to a myriad of roles in the regulation of the electrical excitability of a cell. In neurons, potassium channels have been shown to be involved in controlling the cell resting potential, shape and control action potential duration and frequency, and modulate the efficacy and frequency of neurotransmitter release. Structurally, most potassium channels are formed by an assembly of individual protein subunits defined by at least two transmembrane segments necessary for completing the pore-forming core. The ability of related subunits to mix and form homomeric or heteromeric channels provides additional diversity of function (Hille, 1992).

Cloning and genome projects have revealed numerous genes that encode for potassium channel subunits. Over 80 related potassium channel genes are found in mammalian systems, 90 in worms and approximately 30 in flies (Hille, 1992; Littleton and Ganetzky, 2000; Wei et al., 1996), with a surprisingly high degree of conservation between species. Studies of the mutations in potassium channel genes,

especially in the genetically malleable flies and worms, have revealed the importance of the regulation of the expression patterns of these genes in regulating membrane excitability in different tissues, cell types, and stages of development. Changes in gene expression that alter the density or ratios of channels can have a strong effect on cellular electrical properties, suggesting that the large repertoire of channel genes is not only essential for shaping the electrical characteristics of particular excitable cells but might be also be important in providing dynamic control of the modulation of excitability in response to the environment (Levitan and Takimoto, 1998).

Regulation of excitability as a mechanism for neural homeostasis

Throughout life, neurons are subject to a number of developmental and environmental perturbations that can alter excitability. In recent years it has become evident that neurons have the capacity to respond to the perturbations in synaptic activity, electrical excitability or other neural function in order to maintain their duty within a reasonable physiological range. The mechanisms that mediate the maintenance of neuronal functional properties are by definition homeostatic (Davis and Bezprozvanny, 2001).

The term homeostasis (from Greek: to remain the same) was coined in the 1930's by American physiologist Walter Cannon to describe the capacity of the body to efficiently regulate its internal environment to maintain a stable physiological equilibrium. This extraordinary property of living organisms has intrigued physiologists for many years and has been revealed in a wide range of systems including cellular responses such as to the changes in extracellular osmolarity, and system-wide responses like the regulation of blood pressure (Hohmann, 2002;

Dampney et al., 2002). In the nervous system, homeostatic mechanisms sensitive to absolute levels of membrane depolarization could be used to set reasonable physiological limits beyond which changes in neural activity are not tolerated and prevent neurons from firing excessively or become unresponsive (Davis and Bezprozvanny, 2001).

Since the intrinsic excitability of the nervous system is determined by the combination of ion channels expressed in neurons, regulation of ion channel density has been proposed as a homeostatic mechanism by which neurons can globally modify their activity without interfering with synapse-specific mechanisms of plasticity like long-term potentiation and long-term depression (Turrigiano, 1999; Marder et al., 1996).

Several studies have reported evidence of the modulation of voltage-gated ionic currents in response to long lasting changes in neural activity. In Drosophila studies, blocking synaptic activity by expression of tetanus toxin light chain or mutations that reduce the neurotransmitter acetylcholine, results in an increase in voltage-gated inward Na^+ and outward K^+ currents that promotes neural excitability (Baines et al., 2001). In another study of stomatogastric neurons of crustacea, transition from tonic to bursting activity have been shown to involve an up-regulation of inward Ca^{2+} conductance and a down-regulation of outward K^+ conductances (Turrigiano et al., 1995), while in cultured cortical pyramidal neurons, depriving activity for two days increases sensitivity to current injection by selectively regulating voltage-dependent conductances (Desai et al., 1999).

Concordantly, other studies have shown that changes in the expression of

voltage-gated ion channels result in significant modifications in the electrical properties of neurons. For example in Aplysia, over-expression of an A-type potassium channel exerts a substantial shortening of action potential duration, enhances its hyperpolarizing afterpotential, and depresses the amount of transmitter release, whereas expression of a non-inactivating potassium channel driven by a rat heat shock promoter increases the resting potential of Aplysia silent neurons (Kaang et al., 1992; Han et al., 1999). Meanwhile in Drosophila, increase in ion channel gene dosage by a duplication of the sodium channel gene *para* significantly affects the excitability properties of neurons manifested as an increase in amplitude and duration of excitatory junction potentials (Stern et al., 1990). Additionally, a study in developing inferior colliculus neurons shows that depolarization selectively increases the expression of the Kv3.1 potassium channel resulting in an increase in the amplitude of a high-threshold, noninactivating current (Liu and Kaczmarek, 1998).

Altogether, this evidence suggests that the regulation of expression of voltage-gated ion channels genes is critically important for mediating long-term homeostatic changes to maintain the excitability of the nervous system.

Volatile solvents alter neural excitability

Many drugs have been shown to produce significant alterations in the activity of the nervous system causing severe physiological and behavioral effects. Based on their specific effects on neural function, these drugs can be classified into two general classes. One group is composed of the "sedative-hypnotic drugs" that depress brain function, and include barbiturates, benzodiazepines and volatile solvents, like ethanol, inhalants and general anesthetics. The second group is composed of the

"psicostimulants" that stimulate brain function and include cocaine, amphetamines, caffeine and nicotine (Julien, 2004).

Of all these drugs, the neural depressants and in particular the volatile solvents, present the major intrigue as their mode of action has been shown to compromise a large variety of neural targets and at the same time elicit a wide range of behavioral and physiological responses that range from hyperactivity to profound sedation. Low or moderate exposure to volatile solvents can have excitatory effects, while higher levels of exposure produce sedation and anesthesia. The states of euphoria, dis-inhibition and drowsiness associated with solvent inhalation has led to their use as abused drugs in some cases (Dinwiddie, 1994; Flanagan and Ives, 1994), while the states of sedation, unconsciousness and sleep induced by volatile anesthetic has led to their use a valuable clinical tools (Meiser and Laubenthal, 2005).

Increasing evidence suggests that volatile anesthetics such as chloroform, halothane or enflurane suppress neural function through interactions with voltage- and ligand-gated ion channels (Yamakura et al., 2001; Harris et al., 1995; Shiraishi and Harris, 2004; Duch et al., 1998; Urban and Friederich, 1998). Many abused volatile organic compounds including ethanol share these properties and are thought to induce similar effects to those of volatile anesthetics (Evans and Balster, 1991; Tegeris and Balster, 1994). In general, the mechanisms of action of volatile solvents is mediated by the inhibition of excitatory ligand-gated ion channels permeable to Ca^{2+} and other cations that tend to depolarize and bring the cell to its firing threshold, and by the potentiation of inhibitory ligand-gated ion channels permeable to small anions which cause hyperpolarization of the cell and prevent firing. The combination of these

effects results in significant depression of the nervous system.

Amongst the most common excitatory ligand-gated cation channels inhibited by solvents one finds the nicotinic acetylcholine (nACh) and *N*-methyl-D-aspartate (NMDA)-selective glutamate receptors, while the predominant inhibitory ligand-gated anion channels potentiated by solvents are the gamma-aminobutiric acid type A ($GABA_A$) and the glycine receptors. Additionally, some volatile solvents have also been shown to distinctively affect several other ion channels such as the voltage-gated sodium and potassium channels and the ligand-gated alpha-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA) and 5-hydroxytryptamine$_3$ (5-HT_3) ion channel receptors. The slight difference in protein targets affected by the various solvents is thought to explain some of the differences in the physiological and behavioral responses elicited by these drugs.

Drug tolerance

Based on the alterations in neural excitability resulting from exposure to these drugs, it is expected that changes in activity trigger homeostatic mechanisms that attempt to restore normal neural excitability. Evidence of homeostatic neural adaptations to drug that affect the nervous system is probably manifested in the form of tolerance to these drugs.

A widespread interpretation of drug tolerance is that sustained or repeated exposure to a drug triggers multiple adaptive mechanisms that reduce the effects of a particular drug dose. In many cases, these adaptive processes lead to the need for an increased dose to produce effects of a given magnitude and may also result in the

physiological manifestations of dependence and withdrawal symptoms that underlie addiction.

However, tolerance to a specific drug can be manifested in many different forms depending on the dose, frequency, and duration of administration. Generally, a decrease in sensitivity that develops during the course of a single drug exposure is termed acute tolerance, whereas an acquired decrease in sensitivity to the effect of a drug that results from repetitive exposure to that drug is termed chronic tolerance. Rapid tolerance describes a form of chronic tolerance that develops after a single prior exposure to the drug (Young and Goudie, 1995).

Additionally, different models have been suggested for the mechanisms underlying the different forms of tolerance. A distinction is often made between tolerance that results from increased metabolic clearance of a drug, which reduces the concentration of a drug or its duration of action in a target system (pharmacokinetic tolerance) and tolerance that results from cellular-adaptive mechanisms that reduce the sensitivity of drug-sensitive systems to a given drug concentration (pharmacodynamic or functional tolerance). Homeostatic neural adaptations triggered by the effects of a drug are thought to be a mechanism of functional tolerance (Young and Goudie, 1995).

Because the electrical character of a neuron is an emergent property of the channels that it expresses, likely regulators of these homeostatic changes are ion channel proteins and genes. Changes in gene expression that alter the density or ratios of channels can have a strong effect on cellular electrical properties and could thus contribute to drug tolerance. However, whether the expression of voltage-gated ion

channel genes is modulated by volatile solvents or if tolerance can arise from long-term changes in excitability caused by changes in voltage-gated ion channel gene expression remains unknown.

DISSERTATION OVERVIEW

The overall goal of this study is to investigate the role that the modulation of voltage gated-ion channel gene expression plays in the homeostatic regulation of neural excitability that results in tolerance to sedative drugs. For this purposes, I have used a candidate gene approach in the *Drosophila melanogaster* model system.

Chapters 2 and 3 investigate the effects of volatile solvents on the expression of a candidate voltage-gated ion channel genes and whether the changes in expression play a role in the development of functional tolerance to these drugs. Chapter 2 explores the changes in expression of the calcium-activated, voltage-gated potassium channel gene *slowpoke* in response to alterations in neural excitability, while by using gene mutations and inducible transgenes, chapter 3 investigates the role that transcriptional changes in the *slowpoke* gene play in the development of tolerance to sedation. Although the study focuses on the effects of volatile solvents, a survey of the effects of different agents and treatment that induce changes in excitability has also been conducted to further explore the nature behind the changes observed.

Most of the data presented in chapters 2 and 3 was published in an article in the *Proceedings of the National Academy of Sciences* in December 2004 (Ghezzi et al., 2004) and are reprinted with the copyright permission policy of The National

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Chapter 4 presents data that investigates the role that four other voltage-gated ion channel genes found in Drosophila play in the development of tolerance to sedation. The genes tested encode the *seizure* and *Shaker* potassium channels, the *para* sodium channel and the *cacophony* calcium channel. Additionally, this chapter offers further analysis of the role that two Drosophila signal transduction pathways that modulate activity-dependent changes in gene expression play in tolerance to sedation.

Chapter 5 presents an electrophysiological analysis of the role of the transcriptional regulation of the *slowpoke* gene in modulating neural activity in Drosophila. Additionally, a correlation between the long-term electrophysiological effects of solvent sedation and changes in channel gene expression are presented.

An extensive discussion of the results and further literature review is presented in the Discussion section of each chapter (chapters 2 to 5). Detailed descriptions of the methods used are also presented at the end of each chapter.

Finally, chapter 6 presents a summary of the results obtained along with the conclusions derived.

Chapter 2: Effects of Sedation on Ion Channel Gene Expression

INTRODUCTION

Many organic solvents including ethanol and volatile anesthetics cause dramatic changes in neural excitability. Moderate exposure can have excitatory effects, while higher levels of exposure produce sedation and anesthesia. The euphoria and intoxication associated with solvent inhalation has led to their use as abused drugs (Dinwiddie, 1994; Flanagan and Ives, 1994).

The changes in neural activity caused by exposure to these drugs may trigger homeostatic mechanisms that attempt to restore normal neural excitability. Because the electrical character of a neuron is an emergent property of the channels that it expresses, a likely target of these homeostatic changes are ion channel proteins and genes. Changes in gene expression that alter the density or ratios of channels can have a strong effect on cellular electrical properties (Levitan and Takimoto, 1998) and, in the case of drug-induced changes in excitability, could contribute to drug tolerance and addiction.

The *slowpoke* gene encodes a BK-type Ca^{2+} -activated, voltage-gated K^+ channel. This channel integrates two of the most basic methods of neural signaling: it responds both to increases in free Ca^{2+} and to changes in membrane potential. Null mutations in the *slowpoke* gene cause subtle changes in fly behavior. Drosophila lacking *slowpoke* expression have a reduced capacity for flight, show a stimulus-induced "sticky-feet" phenotype, have an unusual mating song, are arrhythmic with regard to circadian rhythms and are more sensitive to sedation by volatile anesthetics

(Atkinson et al., 2000; Ceriani et al., 2002; Leibovitch et al., 1995; Peixoto and Hall, 1998). In *C. elegans*, the channel encoded by the *slowpoke* homologue is directly modulated by ethanol and genetic studies have shown that it plays a role in the mechanism of intoxication (Davies et al., 2003).

In flies, the *slowpoke* gene is expressed in neurons, muscles, midgut, and trachea and has a very complex transcriptional control region (Becker et al., 1995). Expression of *slowpoke* has been shown to be controlled by *five* tissue-specific promoters which finely determine expression in the different tissues along development (Bohm et al., 2000; Brenner et al., 1996).

In this chapter, I use Drosophila and the channel gene *slowpoke* to study the changes that occur at the transcriptional level in the nervous system in response to alterations in neural excitability. Although this study focuses on the effects of volatile solvents, a survey of the effects of different agents and treatment that induce changes in excitability has also been conducted to further explore the nature behind the changes observed.

RESULTS

Volatile solvents alter excitability in Drosophila

Four different volatile solvents with known effects on behavioral and neurological excitability in mammals were tested in Drosophila. These are: benzyl alcohol, chloroform, toluene and trichloroethylene. Benzyl alcohol is well-known for its properties as a local-anesthetic (Wilson and Martin, 1999; Nuttall et al., 1993) while chloroform is a popular volatile anesthetic widely used the late 19th and early 20th centuries (Wawersik, 1997). Toluene and trichloroethylene on the other hand, are volatile organic compounds commonly used as industrial solvents for glues, paint thinners, degreasing agents, and dry-cleaning agents. When misused, exposure to sufficient concentrations of these compounds results in a range of behavioral disturbances including euphoria and sedation (Flanagan and Ives, 1994; Evangelista and Duffard, 1996). Following is a description of the behavioral effects induced in flies by exposure to each of these solvents.

Benzyl alcohol

Exposure to benzyl alcohol was performed by coating the walls of a 30 ml glass tube with 200 ul of a benzyl alcohol solution in acetone of three different concentrations (0.15%, 0.3% and 0.4%) and a 100% acetone control. The more volatile acetone was then allowed to evaporate as the tubes were continuously rotated in a rotating wheel leaving a thin coat of evenly distributed benzyl alcohol. As flies are placed in the tubes and exposed to the solvent, they rapidly became hyperexcited, and were observed spinning and shaking followed by a period of deep sedation with

no signs of movement or trembling of the legs or wings.

On average, the hyperactive phase appeared at approximately one minute after insertions of flies into the coated tube. Although, initially this was manifested as increased locomotion, this behavior steadily progressed for the next 5 minutes to uncoordinated jumps and spins. After this time, flies dropped to the bottom of the tube, laid on their backs and were unable to right them self up, but were still able to show movement of the limbs (mainly shaking). By the 10th minute, all movement stopped completely. At this point, flies were removed from the benzyl alcohol coated tube and transferred to a clean glass tube.

All the three doses were sub lethal and flies returned to normal behavior after a recovery period. Although the duration of the hyperactive phase did not changed with the different doses, the sedative phase became significantly longer as the dose of benzyl alcohol increased. Flies that were treated with the lowest concentration of benzyl alcohol (0.15%) started recovery within the first two minutes, while flies treated with 0.3% or 0.4% benzyl alcohol remained sedated for approximately 10 and 20 minutes respectively. Recovery was indicated by the return of a natural standing posture. In most cases, individual flies resume normal behavior a few seconds after recovery, characterized by the typical exploratory behavior and climbing of the walls of the tube, a few seconds after recovery.

Chloroform

Since chloroform is significantly more volatile than benzyl alcohol, exposure to this solvent was performed differently. Flies were exposed to the chloroform by inverting a glass tube containing flies over a small centrifuge tube containing 500 ml

100% chloroform at room temperature. A porous piece of paper tissue was used to secure the flies in the tube and prevent them from falling into the solvent. No noticeable effect was observed during the first 10 minutes of exposure to the fumes. After this time, flies started falling to the paper barrier at the bottom of the tube with no signs of hyperactivity. Flies laid on their backs, and were completely sedated. After all flies were knocked down (20 minutes) the tube containing chloroform was removed and flies were allowed to recover. With this treatment, flies remained sedated for at least 10 minutes, after which the first flies started to recover. It took approximately 10 more minutes for the rest of the population to recover fully. However, in almost every case, a small percentage of flies (<10%) never recovered. As with benzyl alcohol, recovery was indicated by the return of a natural standing posture.

Toluene

Flies were exposed to toluene in the same way as to chloroform. As with chloroform no noticeable effect was observed during the initial minutes of exposure to the fumes. In this treatment, effects were evident at around 8 minutes. With this solvent however, a very different effect was observed. A pronounced state of hyperactivity that resembled the effect induced by the initial phase of benzyl alcohol exposure was noticed. Mainly, flies were observed spinning and shaking for at least 20 minutes. After this time flies some flies stopped moving and gave the impression of being dead as indicated by a characteristic positioning of the wings (snapped back) that dead flies usually display. Indeed, this flies never recovered after removal of the solvent.

In a second trial, the same procedure was repeated, but this time, the tube containing toluene was removed 12 minutes after the hyperactive effects initiated. In this trial, flies did not die nor became sedated. The hyperactivity ceased within 2 minutes.

Trichloroethylene

Trichloroethylene exposure was performed in a similar way to toluene. The effects observed were also very similar to toluene exposure, but not as lethal. Again, the solvent was removed after the first 12 minutes of hyperactivity and flies returned to normal behavior.

Benzyl alcohol intoxication increases *slowpoke* mRNA abundance

In spite of the fact that all solvents used induced clear changes in the excitability of the fly's behavior only benzyl alcohol exposure allowed an easy way to control the dose used. Additionally, it was the solvent with the lowest morbidity rates. For these reasons it was chosen to determine if altered neural excitability would cause changes in *slowpoke* gene expression in a dose dependent manner.

To investigated changes in expression caused by exposure to benzyl alcohol, *slowpoke* messenger RNA abundance was measured. Total RNA was extracted from treated flies six hours after solvent exposure, to allow time for changes in gene expression to occur. The level of *slowpoke* mRNA in the nervous system was determined using real-time RT-PCR to quantify the neural-specific *slowpoke* exon called C1 (Brenner et al., 1996).

For this method, total RNA is reverse transcribed using gene specific primers

for C1. The resulting cDNA was then amplified by PCR in the presence of gene specific dual-labeled single stranded probes for C1. As the cDNAs are amplified at each PCR cycle, the dual-labeled probes are destroyed and fluorescence is emitted in a manner proportional to the quantity of the amplified product.

To account for variability in purification efficiency, the abundance of *slowpoke* mRNA was normalized and expressed relative to the abundance of mRNA from the Cyclophilin 1 gene. Cyclophilin 1 mRNA was chosen as an internal control because its abundance was not affected by the experimental paradigm (Figure 2.1B). The validity of this control was confirmed by demonstrating that normalization against total RNA yielded a *slowpoke* expression profile that was essentially the same as that obtained using the Cyclophilin internal control.

Following a single 15 minute exposure to three different doses of benzyl alcohol a dose-dependent change in the abundance of *slowpoke* mRNA was observed (Figure 2.1A). The intermediate dose (0.3%), which produced a 53% change in *slowpoke* message abundance, was chosen for subsequent experiments because it had the largest effect on *slowpoke* mRNA abundance without compromising viability.

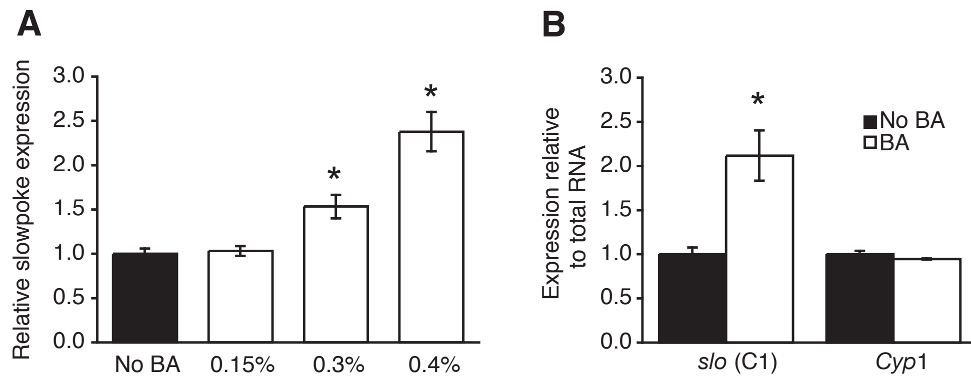

Figure 2.1: Effects of benzyl alcohol on *slowpoke* mRNA levels. Flies were placed in glass vials whose walls were coated with 200 ul of a 0.15% 0.3% or 0.4% benzyl alcohol solution. Sedated flies were allowed to recover for 6 hours and then the abundance of *slowpoke* and *Cyclophilin* transcripts were measured. A) A dose-dependent benzyl alcohol-induced increase in *slowpoke* mRNA level as measured by real-time PCR is observed. The abundance of *slowpoke* message was expressed relative to the *Cyclophilin* internal control. The abscissa shows normalized *slowpoke* mRNA levels relative to control animals (No BA) that were not exposed to benzyl alcohol but were otherwise treated identically. B) Validation of the *Cyclophilin* internal control. The abundance of the *slowpoke* and *Cyclophilin 1* (Cyp1) messages from untreated flies (black bars) or treated with 0.3% benzyl alcohol (white bars) was normalized to total RNA and expressed relative to control animals (untreated). Error bars show standard error of the mean. (*) indicates significant difference from control ($P < 0.01$).

Increase in *slowpoke* mRNA abundance occurs through transcriptional activation of neural promoters

The benzyl alcohol-induced increase in *slowpoke* mRNA could arise from the two different situations. One, in which transcription from the *slowpoke* gene has been activated, leading to the production of new transcripts, and another in which the stability of existing *slowpoke* mRNA is increase, preventing normal degradation of the transcripts.

To determine if the increase in *slowpoke* mRNA abundance arises from transcriptional activation of the gene or from changes in mRNA stability, a transgenic line of flies carrying the P3 reporter-gene construct (figure 2.2A) was used. This construct contains the portion of the *slowpoke* transcriptional control region responsible for neural expression, including promoters/exons CO and C1, driving expression of the β-galactosidase reporter gene. Since flies do not naturally express the β-galactosidase gene, one can detect transcriptional activation of the slowpoke neural promoters by assaying for β-galactosidase activity. In fact, it has been demonstrated that expression from this construct reproduces the neural expression pattern of *slowpoke* (Bohm et al., 2000; Brenner et al., 1996; Thomas et al., 1997).

To test if the *slowpoke* neural promoter is activated by benzyl alcohol, the response of the P3 β-galactosidase reporter transgene was measured in treated and control P3 flies. The endogenous *slowpoke* gene and the reporter gene share the same transcriptional control regions, however they express distinct transcripts whose stability is unlikely to be co-regulated. Therefore, a coincident increase in *slowpoke* mRNA and in *slowpoke* driven β-galactosidase expression is likely to reflect an

increase in promoter activity rather than a change in mRNA stability. P3 flies treated with benzyl alcohol showed a 41.5% increase in β-galactosidase specific activity 11 to 14 hours after treatment, relative to untreated flies (Fig. 2.2B). The increased incubation time, with regard to the previous experiment, was included to ensure enough time for translation of the reporter. This response is similar to the change observed in mRNA abundance from the endogenous gene. Therefore, the benzyl alcohol-induced boost in expression is largely or completely attributable to a change in the transcription rate of the gene.

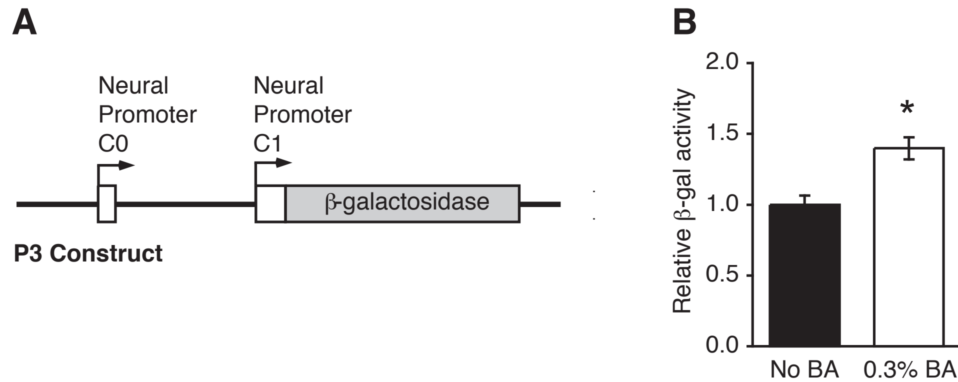

Figure 2.2: Benzyl alcohol-induced increase in *slowpoke* transcriptional activity. Five to seven day old transgenic flies carrying a transgene composed of the *slowpoke* neuronal promoter upstream of the β-galactosidase gene were placed in glass vials whose walls were coated with 200 ul of a 0.3% benzyl alcohol solution. Sedated flies were allowed to recover for 11 to 14 hours and then transcriptional activity of the transgenic *slowpoke* promoter was determined by measuring β-galactosidase specific activity. (A) Map of the P3 transgenic construct in which the *slowpoke* neural promoter drives expression of the β-galactosidase gene. (B) Effects of benzyl alcohol on transcription from the P3 transgene as measured by β-galactosidase specific activity (BA). β-galactosidase specific activity was normalized to total protein and expressed as to untreated control animals (No BA) that were not exposed to solvent but were otherwise treated identically. Error bars show standard error of the mean. (*) indicates significant difference from control ($P < 0.01$).

Increased *slowpoke* expression is a response to sedation

Considering the important role of *slowpoke* in regulating electrical excitability in neurons, it seems reasonable to believe that the observed increase in *slowpoke* expression after benzyl alcohol, a solvent anesthetic that dramatically affects the activity of the nervous system, is a compensatory mechanism that responds to the changes in activity rather than to the solvent it self. However, when exposed to an incapacitating dose of benzyl alcohol, flies pass through a hyperkinetic phase before entering the sedative phase. Thus, the induction of *slowpoke* could be a response to either hyperactivity or sedation. A variety of drugs, temperature treatments and excitability mutants with different effects on the activity of the nervous system were used to determine if the increase in *slowpoke* mRNA is a response to the hyperactive or the sedative phase.

To examine the effect of hyperactivity on *slowpoke* mRNA abundance, genetic and environmental methods were used. First, ion channel mutations were used to produce animals that were genetically hyperactive. In Drosophila, alterations in gene dosage by mutations that target ion channels directly affect the electrophysiological properties of the nervous system and result in altered behaviors. The mutations used were sei^{ts1}, Hk^{1}, $eag^{1}Sh^{120b}$ double mutant and Dp-*para* (Ganetzky and Wu, 1983; Jackson et al., 1985; Kamb et al., 1988; Stern and Ganetzky, 1989; Stern et al., 1990). The first three are mutations in voltage-gated K^+ channel genes or K^+ channel accessory subunits and produce hyperexcitability by perturbing K^+ channel activity. The fifth mutation (Dp *para*) is a duplication of the *para* Na^+ channel gene and is believed to produce hyperexcitability by increasing the

production of *para* voltage-gated Na^+ channels. The mRNA abundance of *slowpoke* in these mutants was determined using the real-time PCR method described previously. In all of these animals *slowpoke* mRNA abundance is reduced with respect to the wild type (Figure 2.3A).

Hyperexcitability without sedation was also environmentally induced. As described previously, in flies, toluene and trichloroethylene, except at extremely high doses, produced only a hyperkinetic response. Additionally, when flies are exposed to a low dose of benzyl alcohol but are removed from the solvent before they become sedated, they show only hyperkinesis. Elevated temperature (37ºC) also causes flies to move more rapidly (Gibert et al., 2001) and was also used to produce populations of hyperkinetic flies. Levels of mRNA were measure 6 hours after each treatment. Each treatment reduced the abundance of *slowpoke* mRNA (Figure 2.3A).

Conversely, sedation without hyperactivity was also induced. Chloroform which as described previously causes sedation, as well as CO_2 and cold anesthesia (0ºC), two common forms to induce sedation in flies (Xia et al., 1999; Seiger and Kink, 1993), were used. Additionally, the sodium channel blocker tetrodotoxin (TTX), was applied by injecting flies with a sublethal dose that causes paralysis. Each of these treatment caused an increase in *slowpoke* mRNA abundance (Figure 2.2B, C).

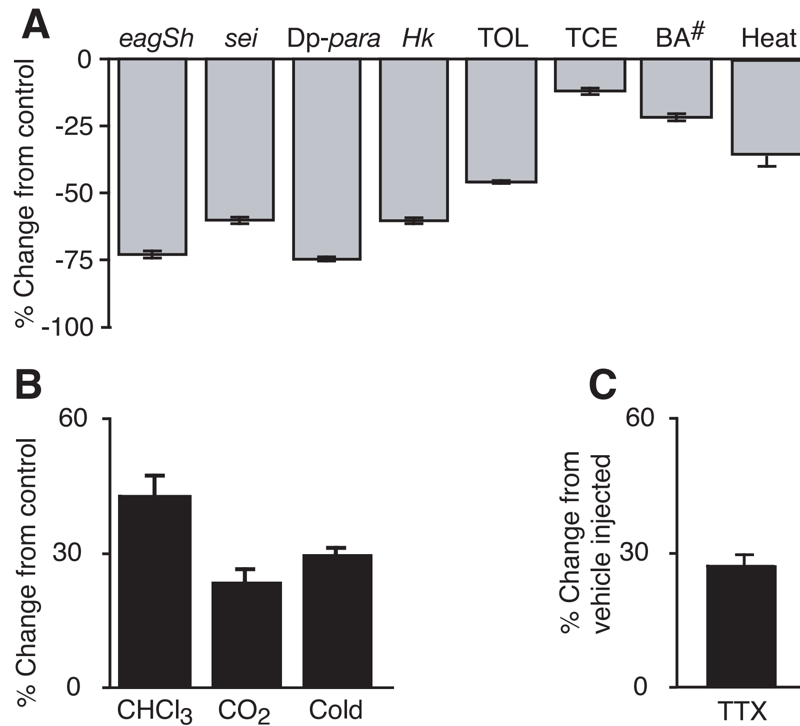

Figure 2.3: *slowpoke* gene expression and activity levels are inversely related. *slowpoke* transcript abundance relative to *Cyclophilin 1* as measured by real-time RT-PCR from mutant flies and flies exposed to drugs that induce hyperactivity (gray bars) or sedation (black bars). A) Relative *slowpoke* expression in the hyperexcitable mutants $eag^{1}Sh^{120b}$, sei^{ts1}, Hk^{1}, and Dp-*para* and in animals treated with toluene (TOL), trichloroethylene (TCE), 0.3% benzyl alcohol (BA#-flies were removed from the benzyl alcohol before entering anesthesia), and 37°C heat shock (Heat). B) Relative *slowpoke* expression levels, six hours after sedation with chloroform ($CHCl_3$), carbon dioxide (CO_2) or 0°C cold anesthesia (Cold). C) Relative *slowpoke* expression levels, six hours after tetrodotoxin (TTX) injection. All values are significantly different from control ($p < 0.01$)

Effects of sedation on *slowpoke* expression are neural-specific and may involve the transcription factor CREB

In order to test if the effects on *slowpoke* expression are tissue specific, I examined the effects of benzyl alcohol and toluene on the mRNA of a muscle-specific isoform of *slowpoke*.

Total RNA was extracted from treated flies six hours after solvent exposure, to allow time for changes in gene expression to occur. The level of *slowpoke* mRNA in muscles was determined using real-time RT-PCR to quantify the muscle-specific *slowpoke* exon called C2 (Brenner et al., 1996). No significant change was observed in *slowpoke* muscle mRNA levels following a single 15 minute exposure to benzyl alcohol or toluene (Figure 2.4A).

Finally, the effects of benzyl alcohol- and toluene-induced changes in activity were also tested on the mRNA levels of the transcription factor CREB. This transcription factor whose name stand for cAMP responsive element binding protein, has been implicated as underlying some of the long-lasting changes in neural gene expression following drug exposure (Chao and Nestler, 2004; McClung and Nestler, 2003). In Drosophila, the *dCrebA* gene encodes the d-CREB-A protein, one of the two CREB-type transcription factor genes found in flies. Following a single 15 minute exposure to a dose of 0.4% benzyl alcohol a change in the abundance of *dCrebA* mRNA was observed. On the other hand, exposure to toluene did not cause a significant change in *dCrebA* (Figure 2.4B), suggesting that up-regulation from this gene responds to sedation and not to hyperactivity.

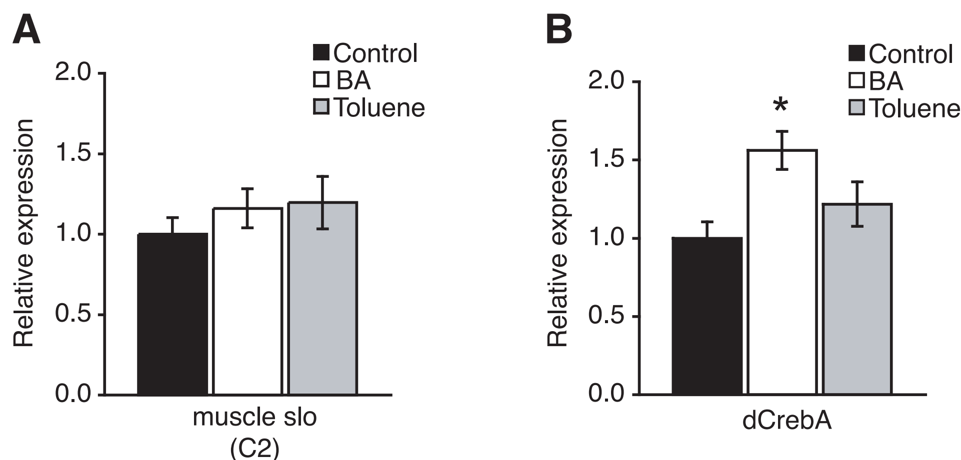

Figure 2.4: Effects of solvents on the expression levels of a muscle-specific *slowpoke* transcript and the *dCrebA* gene. A) The mRNA abundance of a muscle *slowpoke* isoform was measured relative to *Cyclophilin 1* by real-time RT-PCR from wild type flies that were treated with either 0.4% benzyl alcohol (BA), toluene or were left untreated (Control). The abscissa shows normalized mRNA levels from the *slowpoke* muscle C2 exon relative to control animals that were not exposed to solvents but were otherwise treated identically. B) The mRNA abundance of the *dCrebA* gene was measured relative to *Cyclophilin 1* by real-time RT-PCR from wild type flies that were treated with either 0.4% benzyl alcohol (BA), toluene or left untreated (Control). The abscissa shows normalized mRNA levels from *dCrebA* relative to control animals that were not exposed to solvents but were otherwise treated identically. Error bars show standard error of the mean. (*) indicates significant difference from control ($P < 0.01$).

DISCUSSION

Increasing evidence suggests that volatile anesthetics suppress neural function through interactions with voltage- and ligand-gated ion channels (Yamakura et al., 2001) (Harris et al., 1995; Shiraishi and Harris, 2004; Duch et al., 1998; Urban and Friederich, 1998). Many volatile organic compounds including those studied here, share this properties and are though to induce similar effects to those of volatile anesthetics (Evans and Balster, 1991; Tegeris and Balster, 1994). Specifically, the organic solvent benzyl alcohol has been shown to be an effective blocker of sodium channels (Haeseler et al., 2000; Elliott et al., 1987), potassium channels, (Elliott and Elliott, 1997) and nicotinic acetylcholine receptors channels (Bouzat and Barrantes, 1991). Chloroform has been shown to potentiate GABAA and glycine receptors (Beckstead et al., 2001; Beckstead et al., 2000) as well as to activate two-Pore-domain K^+ channels (Patel et al., 1999). Additionally, effects of toluene and trichloroethylene were shown to potentiate GABAA and glycine receptors (Beckstead et al., 2001; Beckstead et al., 2000), enhance serotonin-3 receptor function (Lopreato et al., 2003) inhibit NMDA (Cruz et al., 1998) and nicotinic acetylcholine receptors (Bale et al., 2002) and more recently, effects of toluene and trichloroethylene were demonstrated to affect voltage-sensitive Ca^{2+} channel function (Shafer et al., 2005).

Although the molecular targets affected by these compounds may help explain their sedative properties, here we have observed that in Drosophila exposure to this solvents can cause a series of excitation states that range from just sedation (chloroform), just hyperactivity (toluene and trichloroethylene) or a biphasic response represented as a initial hyperactive stated followed by a sedative state

(benzyl alcohol). Interestingly, these opposing responses have also been noted in mice when exposed to different doses of volatile organic solvents, with lower doses corresponding to states of hyperactivity and high doses correlated to the sedative phase (Bushnell et al., 1985). Presumably, the different behavioral responses arises from independent effects of different concentrations on the various ion channel targets.

In the solvent exposure protocols used in this study, the doses used for toluene and trichloroethylene were not sufficient to induce sedation and were limited to a state of hyperactivity. Unfortunately, higher doses attempted with longer exposure times resulted in lethality. On the other hand, the dose used for chloroform induced just the sedative phase with no hyperactivity. A shorter exposure to chloroform will result in no detectable effect.

This results are not entirely surprising since many volatile solvents, especially those use for anesthesia have dose response curve that become extremely steep around doses that induce behaviors that reflect an anesthetized state (Eger et al., 2001). Therefore, in order to generate a complete survey of the different behaviors elicited by the solvents over a range of concentrations, it will require a fine control over the delivery concentrations of the volatilized solvents. Such control usually requires expensive vaporizers used for clinical anesthesia that were not available in the lab.

Only the low volatility of benzyl alcohol permitted control over the dose, without the need for increased exposure time or for a sophisticated anesthetic machine, to elicit the full range of a characteristic biphasic response. Fortunately, the

slight difference in vapor pressure of chloroform and toluene (or trichloroethylene) elicit opposing behaviors in the present Drosophila study using the same crude delivery methodology.

In this study it was hypothesized that the changes in neural excitability caused by these agents will trigger adaptive mechanisms to counter these effects. One possible way for such adaptation may involve the transcriptional regulation of genes involved in the modulation of neural excitability such as ion channel genes. One such gene is *slowpoke*, which encodes the calcium activated potassium channel.

The response in expression of *slowpoke* after exposure to benzyl alcohol is dose dependent. Interestingly, with the different doses of benzyl alcohol, the duration of the hyperactive phase was not changed, while the sedative phase was significantly prolonged as the dose increased. Therefore, the increasing trend of *slowpoke* expression with the dose is presumed to be in response to the increased duration of sedation.

In accordance with this observations, *slowpoke* gene expression responded differently to the solvents with opposing effects on behavioral activity. In other words, increased transcription is observed after exposure to the sedation by chloroform while a reduction in expression results from exposure to the hyperactivity induced by toluene and trichloroethylene. Consistently, even unrelated treatments that result in comparable behaviors; such as temperature shocks (heat or cold), gaseous compounds (CO_2), or even toxins and mutations that target ion channels; resulted in expression profiles that corresponded to the particular behavior elicited by treatment. This evidence thus points toward a non-specific regulation of *slowpoke* expression in

response to activity rather to a specific drug or drug class.

Activity-Dependent regulation of gene expression has previously been identified in the nervous system and is though to be part of a critically important mechanism to compensate or potentiate sudden changes in neural activity. Most studies have focused on the regulation of immediate early genes (IEGs) and the requirement for the cAMP/Ca^{2+} responsive element binding protein CREB, a transcription factor shown to mediate long-term synaptic plasticity mechanisms (Huang et al., 1994; Sheng and Greenberg, 1990; Bito et al., 1996; Etter et al., 2005). However, output target genes directly involved in the modulation of the electrical properties of neurons are still largely unknown.

Considering that potassium channels exert direct control of the resting membrane potentials of excitable cells like neurons (Hille, 1992), this class of proteins represent reasonable candidates to mediate synaptic plasticity mechanisms that may underlie homeostatic adaptations to the experience-dependent changes in neural activity. Furthermore, many of the transcriptional control regions of potassium channels include the cAMP responsive element (CRE), binding motif for the transcription factor CREB, and have been shown to be up-regulated by cAMP (Mori et al., 1993; Gan et al., 1996; Yao et al., 1996), evidence that further support the candidacy of potassium channels for mediating synaptic plasticity mechanisms specifically through transcriptional activation.

Data presented here clearly suggests that the changes in expression of the large-conductance voltage and Ca^{2+} -dependant K^{+} channel encoded by *slowpoke* may be part of a homeostatic response that attempts to resist the changes in neural activity

caused by the various treatments (Further discussion of the role of *slowpoke* up-regulation in molding neural activity is presented in chapters 3 and 5). Not surprisingly, the changes in *slowpoke* are neural specific, and do not involve regulation of muscle expression. In Drosophila, expression of *slowpoke* in different tissues is controlled by multiple regulatory elements thus expression in one tissue can be regulated independently from the others (Brenner et al., 1996; Brenner and Atkinson, 1996). Furthermore, the Drosophila *slowpoke* transcriptional control region contains at least two canonical CRE sequences, 5'TGACGTCA3', in regions that have been shown to control tissue specific expression (unpublished data).

Although, CREB activity associated with the transcriptional activation of CRE regulated genes is modulated by phosphorylation of specific sites in the CREB protein (Gonzalez and Montminy, 1989; Sheng et al., 1990), in Drosophila a CRE binding site is also found 61 bp upstream from the 5'-most start of *dCrebA* suggesting that, like a number of other transcription factors, dCREB-A may autoregulate *in-vivo* (Rose et al., 1997). Therefore, a coincident increase of *dCrebA* and *slowpoke* mRNAs after benzyl alcohol sedation suggests that the activity dependent regulation of *slowpoke* might be mediated by the activation of dCREB-A that precedes binding of CRE sites within the *slowpoke* and *dCrebA* transcriptional regulation regions.

CREB mediated regulation of *slowpoke* is a very satisfying hypothesis since it's activation is mediated by numerous signaling pathways that may be triggered by changes in neural excitability including protein kinase A (PKA), protein kinase C (PKC) and calcium-calmodulin (CaM) kinase II (Gonzalez et al., 1989). Interestingly, toluene exposure, which causes hyperactivity in flies and a decrease in *slowpoke*

expression, did not cause a change in *dCrebA* mRNA, suggesting that dCREB-A may be activated only in response to signaling pathways that result from the depression and not the enhancement of neural activity and acts as a transcriptional activator of *slowpoke* and not as a repressor of transcription.

One signaling pathway that may account for this observation is the CaM Kinase II pathway which becomes activated by changes in intracellular calcium commonly associated with neural activity. In fact, this Drosophila CREB contains three CaM Kinase II phosphorylation sites but no PKA phosphorylation sites (Smolik et al., 1992).

In summary, the evidence suggests that the changes in activity associated with sedation by volatile solvents and anesthetics result in the up-regulation of the *slowpoke* gene presumably from the activation of the transcription factor CREB by a signaling pathway that senses changes in neural activity. More importantly, these changes may be part of a potassium channel-mediated homeostatic response to sedation.

METHODS

Fly stocks

All flies used in the experiments discussed in this chapter were raised on standard cornmeal/molasses/agar medium. Flies were kept in a room with constant temperature (22 ºC) and 12:12 hrs light:dark cycles. Flies that emerged from pupae were collected over a 2-day period, transferred to fresh food, and studied between three to five days later. In this way, all flies are roughly between five to seven days old. Different mutants with altered excitability were used. Genotype of mutant stocks were $eag^{1}Sh^{120b}$, sei^{ts1}, hk^{1} and Dp-*para*. Transgenic line P3 carrying a reporter gene construct was used for β-galactosidase experiments. Wild types flies used were Canton S and w^{1118} . The w^{1118} flies were tested because it is the genetic background for the transgenic stocks used throughout this study. The behavioral and molecular responses of Canton S and w^{1118} were not distinguishable.

Pharmacological manipulations

Several chemicals were administered to flies to alter their excitability. The following solvents and anesthetics were used: acetone, toluene, trichloroethylene, chloroform, benzyl alcohol (EM Science, Gibbstown, NJ; or Fisher Scientific, Fairlawn, NJ; or Mallinckrodt Baker inc., Paris, KY), CO_2 (Airgas, Radnor, PA), and cold anesthesia. The administration method varied between different chemicals and treatments to accommodate for the differences in their physical properties (figure 2.x).

Exposure to chloroform, toluene, and trichloroethylene was performed by

inverting a 30 ml glass tube containing approximately 25 flies over 0.5 ml of 100% solvent in a 1.5 ml microfuge tube. Flies were trapped in the glass tube by plugging the open end of the tube with a small piece of Kimwipe® (4 cm X 4 cm) wrapped around the cap of a 15 ml conical tube. The cap had been perforated on the top (perforation was 1 cm diameter). The cap and Kimwipe® fitted tightly in the tube. The Kimwipe® is porous enough to let solvent fumes enter the glass tube through the perforation on the cap. After a 15 minute exposure the flies were transferred to food for recovery.

Exposure to benzyl alcohol was performed by coating 30 ml glass vials with 200 ul of a solution of benzyl alcohol in acetone (0.15%, 0.3% or 0.4%). Control experiments were performed using acetone by itself. The vials were continuously rotated for 45 minutes at 22°C to evaporate the more volatile acetone until completion, leaving a evenly distributed thin coat of the less volatile benzyl alcohol. Approximately, Twenty-five flies were placed in each vial and exposed to the benzyl alcohol or to the acetone control (evaporated) until all flies in the benzyl alcohol group were sedated (10 to 15 minutes). After this period, flies were transferred to food containing vials for recovery.

For CO_2 anesthesia, flies were placed in a constant stream of the gas for 15 minutes. Flies were then transferred to food for recovery. Heat and Cold were also used to induce changes in excitability. Glass vials containing approximately 25 flies were placed for 15 minutes in ice for cold anesthesia or in a 37°C incubator for heat shock. In both cases, flies were transferred to food for recovery after treatment. All treatments were done triplicates.

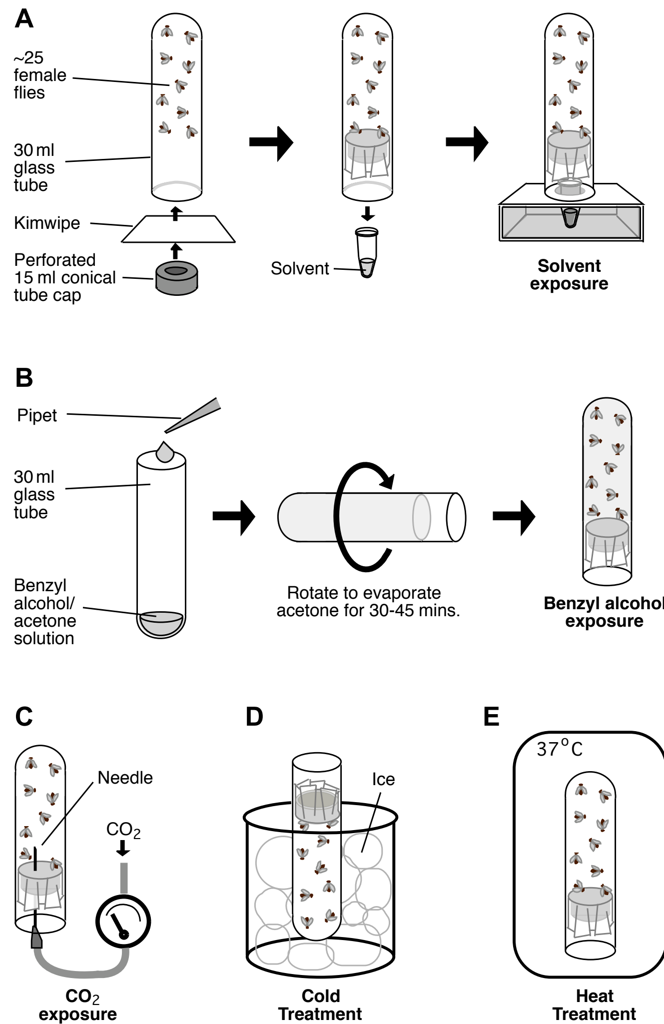

Figure 2.5: Various treatments used to alter excitability in flies. (A) Treatment with highly volatile solvents (toluene, chloroform, trichloroethylene). Flies are trapped in a 30 ml glass vial using a piece of Kimwipe® secured with a perforated cap. The vial is inverted over a centrifuge tube containing the solvent. (B) Exposure to benzyl alcohol. Glass vials are coated with 200 ul of a solution of benzyl alcohol in acetone by rotating the vial at room temperature for 30 to 45 minutes. For exposure, flies are trapped in the coated vial as in A. (C) Exposure to CO_2. Gas is injected at ~10 PSI into a vial containing flies using a needle that perforates the Kimwipe® that traps flies in the vial. (D) Exposure to cold. Flies are trapped in the a glass vial as in A. Vial is immersed in ice. (E) Exposure to heat. flies are trapped in the a glass vial as in A. Vial is placed in a 37ºC incubator.

Finally, the sodium channel blocker tetrodotoxin (TTX) was used to alter electrical activity in fly brains. Flies were injected in the abdomen with 100 nl of TTX dissolved in a vehicle solution 0.1M Na_2HPO_4 and 5mM KCl, pH 6.8. Vehicle alone was used as a control. Approximately 60 flies were injected with the toxin in a period of 30 minutes. Flies were transferred to food right after injection and separated in 3 groups of approximately 20 flies each.

RNA extraction

Total RNA was extracted from each group of 20 to 25 flies six hours after the start of the treatment using a single-step RNA isolation from cultured cells or tissue protocol (Ausubel, 1994) with some modifications.

Flies were ground in liquid nitrogen into a fine powder and transferred to a 1ml dounce homogenizer containing 1 ml of denaturing solution (4M guanidinium thiocyanate, 25mM sodium citrate, 0.1M 2-mercaptoethanol, 0.5% sarkosyl) and homogenized slowly for 2 minutes. 0.5 ml of the homogenate was transferred into a 1.5 ml microfuge tube. 50 ul of 2M sodium acetate, pH 4, 0.5 ml of water-saturated phenol/chloroform 5:1 pH 4.5 (Ambion Inc. Austin, TX) plus 0.1 ml of a 49:1 chloroform : isoamyl alcohol mixture (each from Fisher Scientific, Fairlawn, NJ) were added, mixed and incubated for 15 min in ice. The suspension was then centrifuged for at 4 °C for 15 min in a microcentrifuge at max speed. The upper aqueous phase containing the RNA was transferred to a fresh 1.5 ml microfuge tube. The RNA was precipitated by adding 1 volume of 100% isopropanol, followed by

incubation at –20 °C for 20 minutes and centrifugation for 10 minutes at 10,000xg. The dried pellet was then washed in 0.5 ml 75% ethanol, vortexed, and incubated for 10 to 15 minutes at room temperature to extract residual guanidinium thiocyanate. The pellet was centrifuged for 5 min at 10,000Xg and the supernatant discarded. The pellet was air-dried for 5-10 minutes and resuspended in 0.2 ml DNase buffer, 3 ul of Superase-In RNase inhibitor and 2 ul of RNase free DNase I (Ambion Inc. Austin, TX) and incubated for 30 minutes at 37 °C. Subsequently, the sample was extracted with phenol/chloroform as before followed by an ethanol precipitation. The pellet was then resuspended in 0.1 ml of DEPC – H 2 O and stored at -80 o C. RNA quality was determined by electrophoresis in a 1% agarose gel and quantified by fluorometry using the RiboGreen® RNA quantitation kit (Molecular Probes, Eugene, OR) on a VersaFluor™ fluorometer system (Bio-Rad, Hercules, CA).

Quantitative real-time RT-PCR

The abundance of neurally expressed *slowpoke* message was determined by quantifying abundance of the *slowpoke* exon C1 relative to a *Cyclophilin 1* (*Cyp1*) exon using the TaqMan® real-time RT-PCR assay whereas abundance of the muscle specific *slowpoke* exon C2 and a *dCrebA* exon were quantified relative to *Cyp1* using the SYBR Green I nucleic acid stain real-time RT-PCR assay.

First-strand cDNA was synthesized from 100 ng of total RNA, primed with 200 nM each of gene specific primers (0.2 uM) for neural-*slowpoke* (C1), muscle-*slowpoke, dCrebA* and for *Cyp1* transcripts with Superscript II reverse transcriptase (Life Technologies). Each reaction was performed in triplicate (experimental replicates) from independent RNA samples. Six additional reactions with were

performed from a dilution series of RNA concentrations (0 ng, 25 ng, 50 ng, 100 ng, 200 ng and 400 ng) produced form an arbitrary RNA sample (usually one of the control samples) to create a standard curve. The standard curve is used for quantification purposes and to prove the linearity of the assay.

For experiments involving *slowpoke* exon C1, the cDNA was amplified by real-time PCR in an ABI Prism® 7700 Sequence Detection System (Applied Biosystems) in the presence of gene specific dual-labeled single-stranded probes. The primers used to detect the *slowpoke* exon C1 were 5'- aaacaaagctaaataagttgtgaaagga-3' and 5'-gatagttgttcgttcttttgaatttga-3' while the primers 5'-accaaccacaacggcactg-3' and 5'-tgcttcagctcgaagttctcatc- 3' were used to detect the *Cyp1* message. These primers flanked the TaqMan oligonucleotide probes 5'-(FAM)agaaactgcgcttagtcacactgctcatgt(TAMRA)-3' and 5'-(FAM)cggcaagtccatctacggcaacaagtt(TAMRA)-3' respectively. PCR amplification was performed using Platinum Taq (Life Technologies) in a home-made master mix (1X Platinum Taq buffer, 2 mM $MgCl_2$, 0.15% Triton-X, 1:2000 dilution of Rox dye, 1U Platinum Taq, 0.8 uM dNTP mix). Primer and probe concentration were 0.4 uM and 0.25 uM respectively. The cycling protocol was: 2 minutes at 95°C followed by 40 cycles of a 30 seconds at 95°C denaturing step, a 30 seconds at 60°C annealing step and a 30 seconds at 72°C extension step. Data was collected at every cycle during the annealing step.

For experiments involving *slowpoke* exon C2 and *dCrebA*, the cDNA was amplified by real-time PCR in an ABI Prism® 7700 Sequence Detection System (Applied Biosystems) in the presence of SYBR Green I nucleic acid stain (Molecular

Probes, Eugene, OR). The primers used to detect the *slowpoke* exon C2 were 5'- gctatttataatagacgggccaagt -3' and 5'- ggaaatccgaaagatacgaatgat -3' while the primers 5'- ttcaactacctcagcacctatacga -3' and 5'- tctcgatgtcggagcaaatg- 3' were used to detect the *dCrebA* message. PCR amplification was performed as for C1 but with 0.125 uM SYBR Green I instead of the dual-labeled probe.

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.

β-Galactosidase assay

Transcriptional activity of the transgenic *slowpoke* promoter after treatment was determined by measuring beta-galactosidase specific activity. Protein extract was prepared from P3 flies between 11 and 14 hours after solvent exposure and beta-galactosidase activity determined (Ashburner, 1989). Total protein concentration was determined using the Bradford-based Bio-Rad Protein Assay Kit following the manufacturers instructions. 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.

Chapter 3: Behavioral Responses to Serial Drug Exposure

INTRODUCTION

In the last chapter it was proposed that the perturbation of excitability by solvent sedation would induce homeostatic changes in channel gene expression. A response of this kind should counter the effects of the solvent and if the response was significantly large would reduce drug responsiveness. Although, the effects of sedation with different solvents last no more than 30 minutes, transcriptional responses are presumed to take up to several hours. It is thus predicted that a homeostatic adaptation resulting from changes in gene expression will only become apparent once the flies have recovered from exposure to the solvent.

To test this hypothesis I tested whether a single exposure to benzyl alcohol and the associated increase in the expression from the channel gene *slowpoke* would result in the development of tolerance to a subsequent exposure to this drug. The term "tolerance" is used to identify reduced drug responsiveness that results from prior drug exposure (Chao and Nestler, 2004). Many different drug responses can be used to demonstrate tolerance. Since the observed increase in *slowpoke* expression is directly related to the sedative phase of solvent intoxication, it seems reasonable to test a response that is associated to this phase. The length of time that the animals are sedated is a direct response to the drug. Thus, a drug-induced reduction in the duration of sedation fits the definition of tolerance.

RESULTS

The benzyl alcohol tolerance assay

A common method that has been extensively used to measure sensitivity and tolerance to volatile solvent in flies is the inebriometer. In this apparatus, originally developed by Weber (1988), flies are placed in a cylinder fitted with a series of baffles. When the cylinder is perfused with the vapors of an intoxicated drug, the flies tumble through the baffles and are eluted at the bottom of the of the cylinder. A measure of the mean time required for complete elution of the flies is indicative of the sensitivity of the animals toward the drug. This assay have been successfully used in the study of volatile general anesthetics (Weber, 1988; Krishnan and Nash, 1990; Leibovitch et al., 1995) and ethanol (Moore et al., 1998). However, in this assay, elution time is a result of the initial loss in ability of flies to maintain proper postural control which is due to the drug-induced hyperactivity and not sedation. Since *slowpoke* expression is directly dependent on sedation, this method is not appropriate to test the effects of changes in *slowpoke* expression on sedation.

Therefore, in this study I have used a variation of the "distribution test" that has also been used to test sensitivity to volatile anesthetics (Guan et al., 2000). This test takes advantage of the negative geotactic reflex, which causes flies to climb up the walls of a container. When flies in a vial are exposed to a benzyl alcohol, they become uncoordinated, lose their ability to climb, and fall to the bottom of the vial. They eventually succumb to anesthesia and remain on the bottom of the vial. Recovery from anesthesia can be recorded by monitoring the return of the negative

geotactic behavior.

In order to examine if flies develop tolerance to the effects of benzyl alcohol, naïve flies can be treated with benzyl alcohol at two different moments. Tolerance is said to have been induced if the animals recovered more rapidly from their second benzyl alcohol sedation than from their first sedation (reduced drug responsiveness). In order to allow for transcriptional changes to take effect, exposures to benzyl alcohol are separated from each other by a 24 hour recovery period.

As in every behavioral assay, variability is a major concern. In this assay, a significant source of variability is the changes in humidity, temperature and air pressure that affect the evaporation rates of benzyl alcohol and may result in a dose change between exposures in different days. In order to properly detect changes in recovery from sedation that are a result of tolerance, the doses used in the first and second exposure must be equal. If the dose at the first exposure is larger than the dose at a second exposure, there will be obvious difference in the recovery times from sedation at each exposure that will result in false assessment of tolerance. Unfortunately, day to day variations in atmospheric temperature and pressure, make it impossible to ensure that benzyl alcohol is volatilized in the same way if the exposures are done in different days.

To solve this inconvenience, a population of naïve flies is divided in two groups. On the first day, only one group is treated with benzyl alcohol and the other group is left untreated. Twenty-four hours later, both groups are treated with benzyl alcohol at the same time, thus the dose given to each group is essentially the same. The only difference between groups is that for one group this is their first exposure

while for the other this is their second exposure. Therefore, a difference in the recovery time from anesthesia is a direct measure of the intrinsic sensitivity of each group to the sedative effects of benzyl alcohol.

As second source of variability is of course the natural variations among individuals or populations. To minimize this problem, all flies in each group are age-matched female flies collected from a population reared in the same batch of food.

In order to accurately monitor the duration of sedation of each group, flies are treated in transparent glass tubes. Snapshots are taken every 20 seconds during the course of exposure and recovery with a digital camera under control of a computer. Pictures are then used to analyze the position of flies in the glass tube. Recovery from anesthesia is scored as the return of geotactic behavior. Flies on the walls of the tube are scored as recovered.

Figure 3.1 shows pictures of the same two groups of flies taken a different time-points during the exposure and recovery periods . In each picture, the tube on the left is the control group and received no treatment in a previous day, hence this is its first exposure to benzyl alcohol. The group on the right was treated with benzyl alcohol 24 hours prior to this exposure, therefore this is its second exposure. In this pictures, one can observe that both groups knock-down simultaneously within the first five minutes of exposure. However, when flies are transferred to recovery tubes with no benzyl alcohol (at the tenth minute), the pre-treated group recovers at a faster pace than the control group.

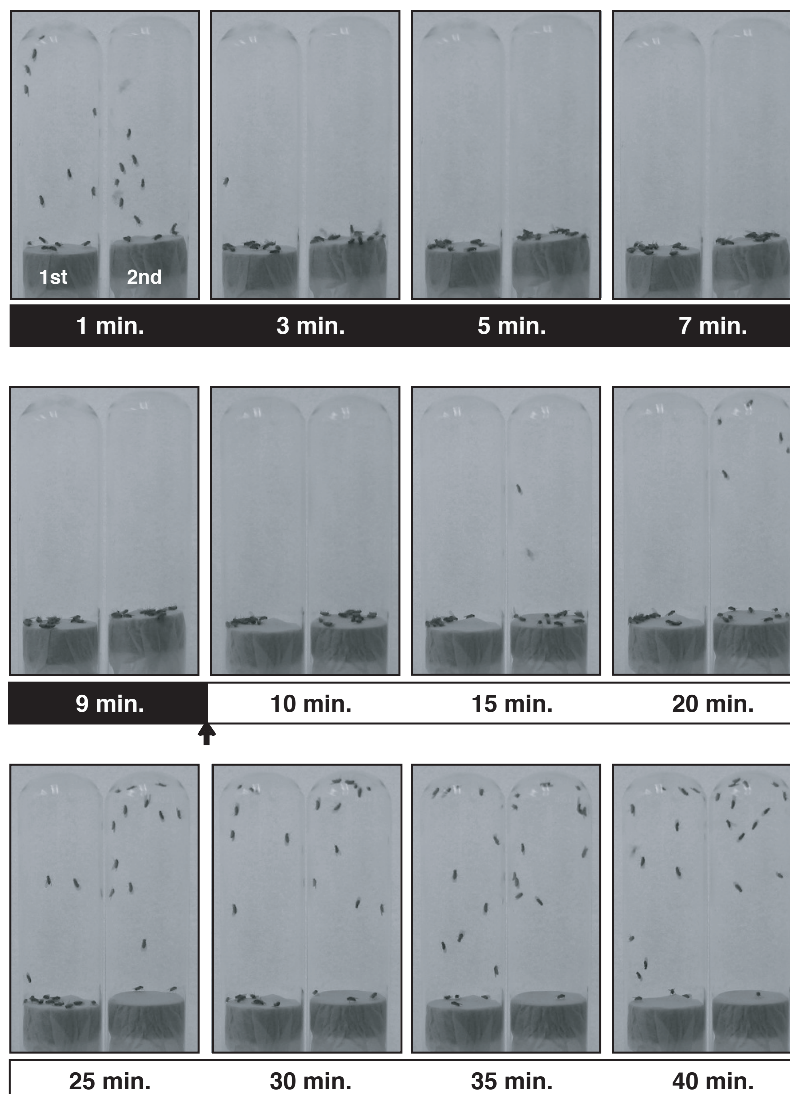

Figure 3.1: Benzyl alcohol tolerance assay. Sequential pictures of two vials of flies taken at different times of exposure and recovery from 0.3 % benzyl alcohol. The vial on the left of every picture, contains flies which have not been treated before, this is their first exposure (1st). For the flies in the right vial, this is their second exposure (2nd). The time point at which each picture was taken is indicated under each picture as minutes after start of the treatment. Time-points depicted over a black bar indicate the presence of the solvent in the tubes while time-points depicted over a white bar indicate that the solvent has been removed. Solvent was removed by transferring flies to clean glass tubes at the end of the ninth minute (arrow).

Benzyl alcohol-induced tolerance is dependent on *slowpoke* expression

It was noticed that a single benzyl alcohol exposure was sufficient to induce tolerance to the drug. Tolerance was said to have been induced if the animals recovered more rapidly from their second benzyl alcohol sedation than from their first sedation (reduced drug responsiveness). Figure 3.2A shows that a population previously treated with benzyl alcohol recovered approximately 5 minutes earlier than naïve flies. The coincident increase in *slowpoke* mRNA levels with the induction of tolerance prompted us to determine if the increase in *slowpoke* expression contributes to tolerance. For this, the responses of the slo^4 mutant were examined. The slo^4 mutation is a chromosomal rearrangement that has a breakpoint in the *slowpoke* gene. This mutation eliminates the production of channels from the gene (Becker et al., 1995; Atkinson et al., 1991). Surprisingly, slo^4 homozygotes are healthy and fecund (Atkinson et al., 2000). The slo^4 flies were subjected to the tolerance test described above. Figure 3.2B shows that in the absence of *slowpoke* expression, prior benzyl alcohol sedation does not induce drug tolerance. Instead, the previously treated slo^4 flies appeared more sensitive and showed a slower recovery from anesthesia when exposed to benzyl alcohol a second time.

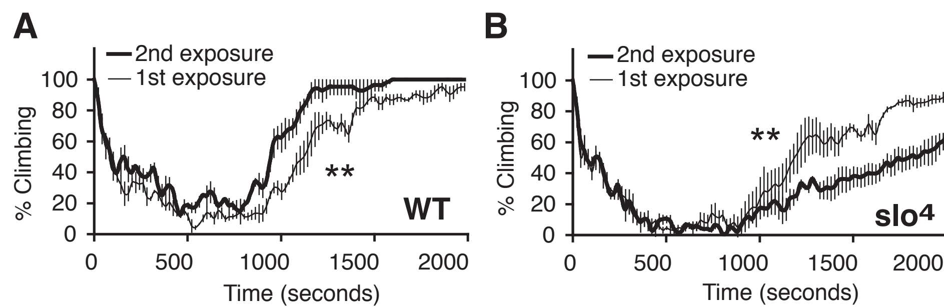

Figure 3.2: Behavioral response to serial benzyl alcohol exposure. Behavioral response to serial benzyl alcohol exposure. Knock down and recovery curve of wild-type (A) and *slo*^{4} mutant flies (B) after one (thin line) and two (thick line) exposures to 0.3% benzyl alcohol. Time between exposures is 24 hours. Loss of climbing indicates sedation. Values are plotted as a percentage of climbing flies against time at 20 second intervals. The double asterisk indicates significant difference from control as determined by the Log-Rank test (n = 3, $p < 0.01$). Error bars are SEM for each data point.

Expression level of *slowpoke* is a predictor of drug sensitivity

To determine whether the level of *slowpoke* expression affects benzyl alcohol responsiveness I manipulated *slowpoke* expression and measured drug responsiveness. Expression was increased by using an inducible *slowpoke* transgene or with drugs, and expression was reduced with environmental treatments.

First, I tested the benzyl alcohol response of flies carrying a heat-inducible *slowpoke* transgene. These animals are homozygous for the slo^4 null allele and carry the B52H transgene. B52H has an hsp70 promoter that drives expression of a *slowpoke* cDNA (Atkinson et al., 1998) (Figures 3.3A and B). In this paradigm, heat shock (37°C for 30 minutes) was used to induce the transgene and was substituted for the first benzyl alcohol treatment. Twenty-four hours later the animals were sedated with benzyl alcohol and their recovery time compared to age-matched B52H siblings that were not heat shocked but that were otherwise treated identically. Heat shocked flies were more resistant to benzyl alcohol and recovered faster than their non-heat shocked siblings (Figure 3.3C). Real-time RT-PCR demonstrated that the heat pulse induces expression from B52H (Figure 3.3B).

To determine if inducible resistance was merely a heat shock response that was unassociated with the B52H transgene, I monitored, in parallel, the effect of heat shock on wild type flies that do not carry the transgene. In wild type flies, heat shock did not induce resistance but slowed the recovery from anesthesia (Figure 3.3D).

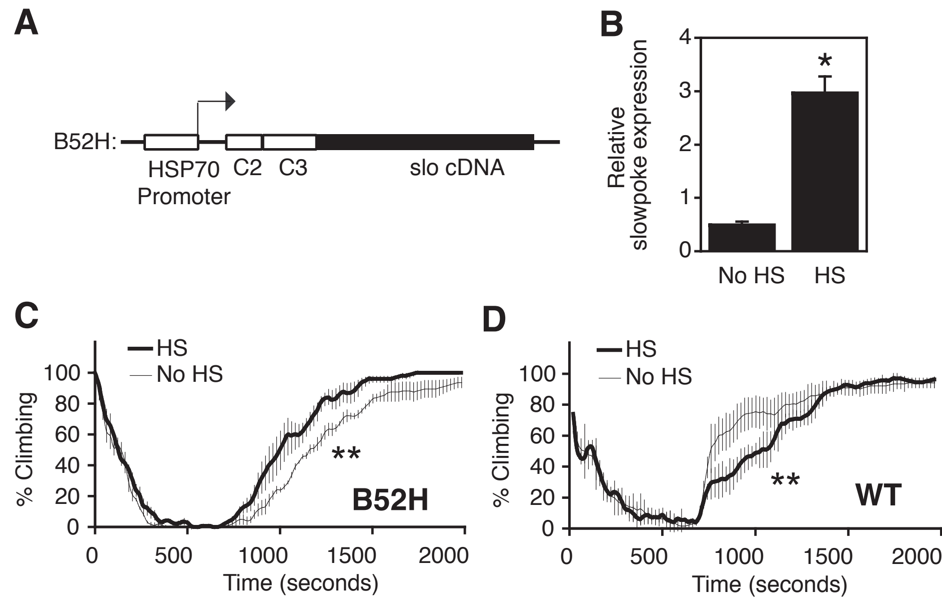

Figure 3.3: Effects of *slowpoke* induction on recovery from benzyl alcohol sedation. A) Map of the B52H transgenic construct (Atkinson et al., 1998) in which the HSP70 promoter drives expression of a *slowpoke* cDNA. C2 and C3 are two exons that are also shown in figure 1D. B) Relative *slowpoke* expression levels in B52H transgenic flies, six hours after treatment with heat shock (HS) or control (No HS). An asterisk indicates significant difference from control ($p < 0.01$). C and D) Recovery curve of wild-type and the HSP70-*slowpoke* transgenic flies (B52H) after exposure to 0.3% benzyl alcohol. Effects of *slowpoke* induction on the recovery were determined by treating the transgenic and wild type flies with a heat shock (thick line) 24 hours before the benzyl alcohol treatment or with no heat shock (thin line). Loss of climbing indicates sedation. The double asterisk indicates significant difference from control as determined by the Log-Rank test ($p < 0.01$). Error bars are SEM for each data point.

A working hypothesis that accounts for these data is that sedation induces *slowpoke* expression and increased *slowpoke* expression results in tolerance (inducible-resistance). In concert with this hypothesis are the results of a dose-response study using benzyl alcohol. Low doses that fail to sedate the animals (0.15%) and do not cause an increase in *slowpoke* expression (Figure 2.1A) also fail to induce drug tolerance (Figure 3.4A). On the other hand, higher doses that induce sedation and *slowpoke* expression also cause tolerance.

In a second test I determined the effects of chloroform-induced *slowpoke* expression on the recovery rate from benzyl alcohol sedation. Chloroform which sedates animals without causing them to pass through a hyperactive phase also produces an increase in *slowpoke* expression (Figure 2.3). A single chloroform sedation was sufficient to induce benzyl alcohol resistance. Figure 3.4B shows that a population previously treated with chloroform recovered from benzyl alcohol sedation more rapidly than naïve flies.

To determine the effect of decreasing *slowpoke* expression I relied on environmental treatments that reduce *slowpoke* mRNA levels. Hyperkinetic behavior induced with increased temperature (37°C) or by exposure to a non-sedating dose of toluene was used to reduce *slowpoke* expression (Figure 2.3). The benzyl alcohol sensitivity of the animals was measured twenty-four hours after exposure. Both the heat pulse (Figure 3.3D) and toluene exposure (Figure 3.4C) not only reduce *slowpoke* mRNA abundance but also cause increased sensitivity to benzyl alcohol.

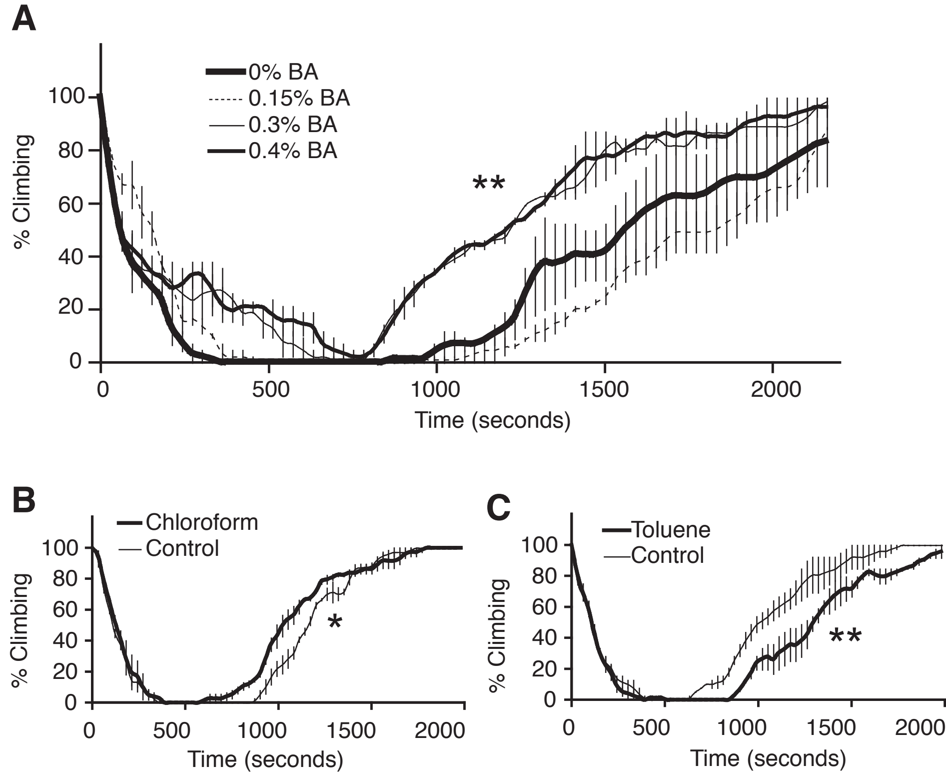

Figure 3.4: Solvents that change *slowpoke* expression alter drug responsiveness. A) Recovery of wild-type flies from 0.3% benzyl alcohol after pre-treatment with 0.15%, 0.3% or 0.4% benzyl alcohol compared to non-pre-treated control flies. B) Recovery of wild-type flies from 0.3 % benzyl alcohol sedation after pre-treatment with chloroform compared to non-pre-treated control flies. C) Recovery of wild-type flies from 0.3 % benzyl alcohol sedation after pre-treatment with toluene compared to non-pre-treated control flies. The asterisk indicates the recovery curves are significantly different from control (* = $p < 0.05$; ** = $p < 0.01$). In panel B it indicates that the 0.3 and 0.4% pre-treatments are significantly different (** = $p < 0.01$) from control (whereas the 0.15% pre-treatment is not). Error bars are standard error of the mean calculated for each data point.

Sedation with non-solvents has also been shown to cause an increase in *slowpoke* expression. I wished to know if non-solvent-induced sedation could also induce benzyl alcohol resistance. Carbon dioxide and cold sedation both induced *slowpoke* expression in the absence of a hyperactive phase. But neither CO_2 (Figure 3.5A) nor cold anesthesia (not shown) changed benzyl alcohol sensitivity. These treatments induce less *slowpoke* expression than benzyl alcohol or chloroform (Figure 2.3B). They may not be sufficiently strong inducers of *slowpoke* expression to cause benzyl alcohol resistance in normal animals. Therefore, I asked if any evidence of CO_2-induced benzyl alcohol resistance could be detected in flies sensitized to benzyl alcohol anesthesia. Flies heat shocked to increase sensitivity were immediately sedated with CO_2. Interestingly, the CO_2 treatment eliminated all evidence of heat-induced sensitization (Figure 3.5B and 3.5C). This evidence suggests that CO_2 sedation does increase benzyl alcohol resistance but that this effect can be detected only in a sensitized background.

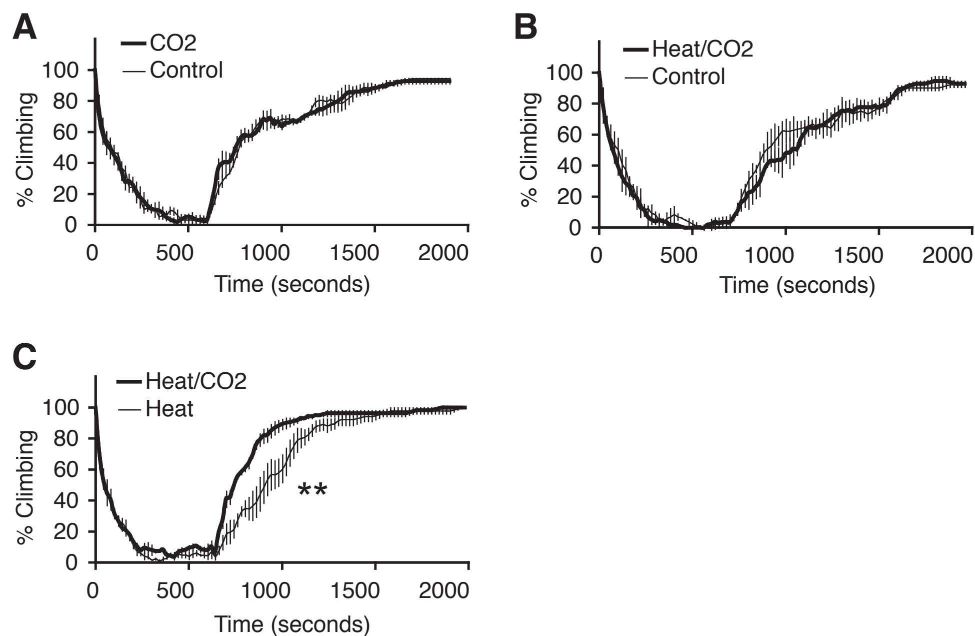

Figure 3.5: Non-solvent treatments that change *slowpoke* expression alter drug responsiveness. A and B) Recovery of wild-type flies from 0.3 % benzyl alcohol sedation after pre-treatment with CO_2 (A) or heat followed by CO_2 (B) compared to non-pre-treated control flies. C) Recovery of wild-type flies from benzyl alcohol after pre-treatment with heat and CO_2 compared to flies pre-treated with heat. The asterisks indicates the recovery curves are significantly different from control ($p < 0.01$). Error bars are standard error of the mean calculated for each data point.

Neural-specific expression of *slowpoke* is necessary for drug tolerance

These studies have shown that up regulation of a neural-specific *slowpoke* transcript is correlated with the appearance of benzyl alcohol tolerance and that a *slowpoke* loss-of-function mutation prevents the acquisition of tolerance. However, this loss-of-function mutation eliminated *slowpoke* expression in all tissues (neural, muscular, epithelial). To demonstrate that tolerance was dependent only on neural-expression of *slowpoke* the trans-heterozygotes mutant $ash2^{18}/slo^{4}$ was used. The $ash2^{18}$ allele is a chromosomal deletion that removes the two transcriptional promoters that drive *slowpoke* neural expression. The promoter that drives expression in muscle is still present (Atkinson et al., 2000). The $ash2^{18}$ allele is a recessive lethal mutation because it also removes the *ash*2 (developmental) gene. Therefore, I used $ash2^{18}/slo^{4}$ trans-heterozygotes. Atkinson *et al.* (Atkinson et al., 2000) have shown that this double mutant has specifically lost expression in the nervous system. These flies were unable to acquire benzyl alcohol resistance (Figure 3.6A) whereas the slo^{4}/ + heterozygotes did acquire resistance (Figure 3.6B).

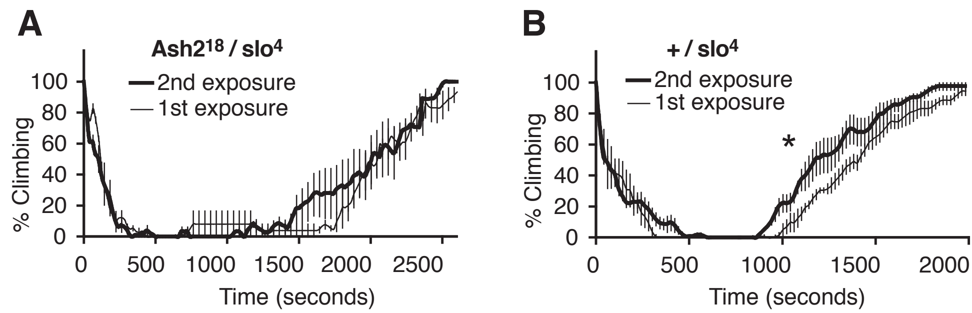

Figure 3.6: Neural-specific expression of *slowpoke* is necessary for induced drug resistance. A and B) Recovery curve of the *ash*2^{18}/*slo*^{4} trans-heterozygous (A) and *slo*^{4}/+ heterozygous flies (B) after one (thin line) and two (thick line) exposures to 0.3% benzyl alcohol (24 hours between exposures). Values are plotted as a percentage of climbing flies against time at 20 second intervals. An asterisk indicates significant difference from control (n=3, $p < 0.05$). Error bars are standard error of the mean calculated for each data point.

Discussion

The term tolerance refers to reduced drug responsiveness that results from prior drug exposure. Tolerance produced by a single brief exposure is termed "rapid tolerance" (Scholz et al., 2000). Large-conductance calcium-activated potassium (BK) channels, encoded by *slowpoke*, participate in regulating neuronal firing patterns and neurotransmitter release. In flies, anesthetization with benzyl alcohol induces both rapid tolerance and increased expression from the *slowpoke* Ca^{2+}-activated K^{+} channel gene. Benzyl alcohol sedation does not evoke this response from the *slowpoke* muscle-specific transcript following sedation. Results presented here indicate that the sedation-induced increase in *slowpoke* expression contributes to acquired benzyl alcohol tolerance.

Scholz *et al.* (2000) have studied Drosophila rapid tolerance as induced by ethanol sedation. They characterized tolerance by measuring the time or dose necessary to sedate the animals. Sedation was defined as the point at which flies lost postural control in an inebriometer or when the flies ceased movement. Using these measures they showed that a single bout of ethanol sedation induces rapid tolerance. They also demonstrated that a mutation interfering with octopamine biosynthesis reduced the acquisition of rapid tolerance by 50%.

In this study I chose to use benzyl alcohol as a model solvent because it was extremely easy to deliver and was extremely well tolerated by flies. With this anesthetic, flies could be repetitively sedated without a loss of viability. For the measurements of rapid tolerance I used a variation of the distribution test (Guan et al.,

2000) in which a human observer records not the knock down time, but the period of sedation. The specific hallmark of recovery that we scored was the resumption of wall climbing. This means that the flies are not merely ‘conscious’ but that they are sufficiently coordinated to implement a rather complex behavior.

I postulate that the benzyl alcohol-induced changes in *slowpoke* expression reflect a homeostatic mechanism that resists untoward changes in net cellular excitability. If hyperexcitability is induced, the proposed mechanism alters channel expression to reduce this excitability, whereas if cellular excitability is suppressed, channel gene expression changes to enhance excitability. This hypothesis does not require that the activity of the *slowpoke* channel be directly affected by this solvent. However, it has been shown that anesthetics and ethanol directly affect *slowpoke* channel activity (Denson et al., 1996; Namba et al., 2000). In some cells, the solvents inhibit channel activity while in others they potentiate the current.

Benzyl alcohol exposure results in an initial hyperkinetic phase followed by a sedative phase. In the previous chapter, I have shown that the hyperkinetic phase reduces *slowpoke* mRNA abundance and causes the drug sensitization. Conversely, the sedative phase stimulates an increase in *slowpoke* expression and induces drug tolerance. I propose that decreased *slowpoke* expression reduces the net excitability of the nervous system while increased *slowpoke* expression enhances neural excitability. Consistent with these interpretations are the observations that mutations that prevent *slowpoke* expression interfere with the acquisition of tolerance while artificial enhancement of *slowpoke* expression with a transgene causes a tolerant-like phenotype. This observation indicates that the changes in *slowpoke* channel

expression make a significant contribution to behavioral tolerance to benzyl alcohol intoxication.

Upon a cursory consideration, these responses appear not to be homeostatic in nature but to exacerbate the changed excitability of the nervous system. That is, generically, one assumes that increased K^+ channel activity would hyperpolarize the cell and reduce excitability while reduced K^+ channel activity would interfere with the ability of the cell to terminate an electrical impulse and thereby enhance electrical excitability. This role of *slowpoke* channels is well documented (Gribkoff et al., 2001; Orio et al., 2002; Sun and Dale, 1998). However, there is substantial evidence that *slowpoke* channel activity can also affect neural excitability in the opposite manner.

Warbington *et al*. (1996) have shown that loss of the *slowpoke* (BK) current in flies leads to reduced motoneuron excitability and neurotransmitter release. It was proposed that these changes occurred because the normal role of the *slowpoke* current was to ensure rapid repolarization of the nerve terminal and, in the absence of the *slowpoke* current, the increase in depolarization led to inactivation of voltage-gated calcium channels. In a study in mammalian anterior pituitary cells it has been shown that BK channel activation prolongs action potential duration, thus facilitating extracellular Ca^{2+} entry (Van Goor et al., 2001). In this study, the authors show that fast activation of BK channels by Ca^{2+} prevents the complete repolarization of the cell by limiting the activation of voltage-gated potassium channels. This generates a plateau potential which results in high-amplitude Ca^{2+} transients. Additionally, Lovell and McCobb (2001) have shown that an increase in the open-probability of BK channels enhances repetitive firing in chromaffin cells presumably by limiting

activity-dependent inactivation of Ca^{2+} and Na^+ channels. Finally, Pattillo *et al.* (2001) have found that toxin blockade of BK channels in Xenopus nerve-muscle synapses cultures significantly decreased transmitter release. They propose that this decrease occurs because of the effect of the BK current on the driving force for Ca^{2+}. In all of these examples, an increase in the instantaneous excitability of the cell leads to a reduction in long term or net excitability. Clearly, an increase in Ca^{2+}-activated K^+ channel expression could elicit a positive rather than a negative effect upon synaptic excitability and conversely, a reduction in *slowpoke* expression could reduce neural excitability.

Regardless of the mechanism, the data clearly suggests a role for the modulation of *slowpoke* gene expression in the homeostatic regulation of excitability. It is not suggested that *slowpoke* expression is responsible for all facets of tolerance. This data indicates only that, in flies, *slowpoke* expression is required for the acquisition of tolerance and that increased *slowpoke* expression can result in a degree of benzyl alcohol resistance. It is likely that *slowpoke* is one component of an orchestrated response of transcriptional changes and post-transcriptional modifications that involves many genes.

Recently, Davis *et al.* (2003) demonstrated that, in *C. elegans, slowpoke* loss-of-function mutations cause ethanol resistance and that *slowpoke* over-expression produces a phenotype that mimics ethanol intoxication. It is important to note that *slowpoke* may play an opposing role in *C. elegans* with regard to its response to drugs. I do not believe that the source of the difference is the choice of drugs. I propose that, in Drosophila, the tolerance mounted to benzyl alcohol and to ethanol

are related phenomenon because exposure to ethanol induces cross-tolerance to benzyl alcohol (data not shown). The work in *C. elegans* focuses on resistance, *per se*, and not on acquired tolerance. It seems unlikely that the origins of resistance and acquired tolerance are sufficiently different to be influenced in opposing manners by the same gene. A study by Leibovitch *et al*. (1995) has also suggested that there exists a positive correlation between *slowpoke* expression and solvent anesthetic sensitivity. This group demonstrated that a null mutation in *slowpoke* increased the sensitivity of flies to halothane, chloroform and trichloroethylene. It is likely, therefore, that the apparent differences in the role of *slowpoke* in the *C. elegans* and Drosophila drug response has to do with the fundamentally distinct organization of the nervous system of these animals. The most important consideration, is not the differences in how *C. elegans* and Drosophila respond to changes in *slowpoke* expression, but in the fact that studies in two very different organisms implicate *slowpoke* as having an important role in the response to sedation.

METHODS

Fly stocks

Drosophila stocks used in this study were Canton S (wild type), B52H, w^{1118}, slo^4, $+/slo^4$ and the $ash2^{18}/slo^4$ trans-heterozygote. 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 (Atkinson et al., 1998). The B52 transgene is constitutively active but is substantially induced by a brief heat shock. Flies were raised on standard cornmeal/molasses/agar medium at room temperature (25°C) in a room with circadian control of illumination (12 light, 12 hours dark). Flies that emerged from pupae were collected over a 2 day period, transferred to fresh food, and studied three to five days later.

Benzyl alcohol tolerance assay

In the first exposure, flies were treated in triplicate with benzyl alcohol (0.3%) or vehicle (100% acetone – evaporated to dryness) as described in Chapter 2. Twenty-four hours later, treated and control flies were simultaneously exposed to benzyl alcohol until all flies were sedated (approximately 10 minutes). At this point flies were transferred to a solvent free glass vial for recovery. 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. The data and statistics shown in the figures are derived from a protocol in which each assay is performed in triplicate. The entire protocol has been repeated, in triplicate, a minimum of three times. Each repetition yielded the same results.

For cross-tolerance or heat-shock experiments, a groups of flies were pre-treated in triplicate with either chloroform, toluene, CO_2, or heat-shock as described in Chapter 2 and a control group was left untreated. Twenty-four hours later, treated and control flies were simultaneously exposed to benzyl alcohol. Exposure and recovery was monitored and recorded as explained above for benzyl alcohol tolerance.

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 (Hosmer et al., 2002). In all of the tolerance assays we 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). However, in behavioral assay we have also included error bars that represent the standard error of the mean for each individual data point.

Quantitative real-time RT-PCR

Total RNA was extracted from 25 B52H flies 6 h after the start of the heat shock or mock treatment by using a single-step RNA isolation tissue protocol as in Chapter 2. mRNA expression from the B52H transgene was determined by

quantifying abundance of the *slowpoke* exon C3 (found in the transgene) relative to a *Cyclophilin 1* (*Cyp1*) exon using the SYBR Green I nucleic acid stain real-time RT-PCR assay as in Chapter 2. The primers used to detect exon C3 were 5' -ttggccgacgatccaaca- 3' and 5' -accagtacttgcgcaccttga- 3'. Each PCR was performed in triplicate, and the yields thereof were expressed as an average mRNA abundance was quantified by using the standard curve method. Significance was calculated by using Student's t test.

Chapter 4: Ion Channels, Memory, Sleep and Tolerance

INTRODUCTION

Although the evidence presented so far implicates that in flies, *slowpoke* expression is required for the acquisition of tolerance and that by itself increased *slowpoke* expression can result in a degree of benzyl alcohol resistance, it is likely that *slowpoke* is one component of an orchestrated response of transcriptional changes and post-transcriptional modifications that involves many genes. In this chapter, I have examined the role that other neurally expressed genes play in the observed activity-dependent manifestations of tolerance to sedation by volatile solvents in flies.

One obvious candidate group to test is composed of other voltage-gated ion channel genes that, like *slowpoke*, are directly involved in shaping the electrical properties of neurons. Changes in the expression of these channels can affect cell resting potentials, action potential threshold and duration, repetitive firing, neurotransmitter release and if co-regulated with *slowpoke* in response to volatile solvents may also participate in the acquisition of tolerance to these drugs.

In almost every case, voltage-gated ion channel genes found in Drosophila have a mammalian homologs with striking similarities in sequence and function. Two voltage-gated Na^+ channels genes have been identified in Drosophila, *para* and DSC. Both genes are widely expressed in the nervous system and have been shown to be essential for action potential propagation. Several voltage-gated K^+ channels from the various mammalian subfamilies have also been found in Drosophila. Apart from

slowpoke, which is the only BK-type channel encoded in Drosophila, there are at least five Kv type channels involved in action potential repolarization; one KCNQ type, associated with heritable human disorders; three EAG (one *eag*, one *elk*, and one *erg*), mediating rapidly activating delayed rectifier K^+ currents (IKr); three inward rectifying Kir type channels, involved in both neuronal excitability and K^+ transport; and eleven TWIK K^+ channels, which are modulated by a number of factors, including pH, small signaling molecules, and membrane tension. Additionally, there are at least four genes encoding voltage-gated Ca^{2+} channels, including homologs of the mammalian T-type/α1G, L-type/α1D, and N-type/α1A genes (Littleton and Ganetzky, 2000).

Although highly desirable, testing all of these genes represents a difficult task to accomplish in a single study especially since mutants for all these genes are not readily available. The ion channels tested here are: the well studied voltage-gated sodium channel gene *para* (Stern et al., 1990), the L-type voltage-gated calcium channel *cacophony* (*cac*) (Smith et al., 1996), and two voltage-gated potassium channels: the A-type Kv channel *Shaker* (*Sh*) (Tempel et al., 1987) and the Drosophila homolog of the human ERG *seizure* (*sei*) (Wang et al., 1997). Mutations in these channels result serious alteration in neural excitability. Generally, mutations that affect sodium or calcium channel function results in under-excitable cells, whereas perturbations in potassium channel function results in hyperexcitability.

Another candidate group is that of genes involved in signaling pathways that mediate activity-dependent changes in gene expression. One of the most studied and best characterized neural signaling pathways that have been directly involved in

activity-dependent behaviors, in both Drosophila and mammalian systems, is the cAMP/Ca^{2+}-CREB pathway. CREB (cAMP/Ca^{2+} Response Element Binding protein) is a transcription factor activated by multiple protein kinases, including the cAMP-dependent PKA, and several Ca^{2+}-dependent protein kinases, such as the calcium/ calmodulin-dependent protein kinase type II (CaMKII) (Gonzalez et al., 1989; Shaywitz and Greenberg, 1999). Gene regulation by CREB has been shown to participate in learning and memory (Silva et al., 1998), in the cellular responses to drugs of abuse (Nestler, 2004) and in the circadian regulation of behavioral activity (Kako and Ishida, 1998).

Furthermore, in Drosophila the *slowpoke* and the *dCrebA* transcriptional control regions contain CREB response elements, and both genes are simultaneously up-regulated after benzyl alcohol sedation (Chapter 2). However, the signaling pathways that couple changes in neural activity with the up-regulation of *slowpoke* remain unknown. Studying the effects of manipulating genes involved in other CREB mediated activity dependent behaviors on the phenomenon of tolerance may offer clues into deciphering of the signaling pathways involved in this response.

Although multiple genes have been identified to be involved in learning and memory and circadian rhythms, the *dunce* (*dnc*) and *period* (*per*) genes play central roles in each of this pathways respectively and appear to be excellent tools to investigate the involvement of CREB mediated signaling pathways in tolerance to sedation. On one hand, *dunce* encodes a cAMP-specific phosphodiesterase that when mutated, it has been shown to interfere with learning in several of the conditioning tests involving odors and electric shocks or sugars (Dudai et al., 1976; Aceves-Pina et

al., 1983; Tempel et al., 1983). On the other hand, *period* encodes a PAS domain protein that is essential for biological clock functions and determines the period length of circadian and ultradian behavioral rhythms of activity(Reddy et al., 1984). Mutations in *per* cause flies to have aberrant circadian rhythms including eclosion and locomotor activity (Konopka and Benzer, 1971).

Although these two genes do not have a direct involvement in the electrical properties of cells, they are part of signal transduction pathways that modulate gene products and the expression of genes involved in synaptic plasticity. I have used mutants in *dnc* and *per* to examine the effects of disruptions of these two pathways in tolerance to sedation.

RESULTS

To examine the implications that other genes have in tolerance, I have followed a similar methodology to the one used in the previous chapters for *slowpoke*. First, the expression patterns of the various ion channel genes mentioned above was tested by quantification of their transcript abundance in response to benzyl alcohol-induced sedation or toluene-induced hyperactivity. Secondly, I tested mutants in three of these ion channel genes and in the two signal transduction genes to determine if expression of their gene products is necessary for tolerance to benzyl alcohol or if any change in excitability caused by these mutations alters in any way the phenomenon of tolerance. Expression patterns of *period* and *dunce* were not examined since these are expected to mediate gene transcription events rather than be regulated themselves.

Expression of other ion channels is not affected by solvent-induce changes in neural activity

The mRNA levels for *para, Shaker, seizure* and *cacophony* was measured in flies six hours after treatment with benzyl alcohol and toluene using real-time RT-PCR. Surprisingly, these treatment did not cause significant changes in the abundance of the *Shaker* or *seizure* voltage-gated K^+ channel mRNAs, the *para* voltage-gated Na^+ channel nor in the voltage-gated Ca^{2+} channel *cacophony* (Figure 4.1).

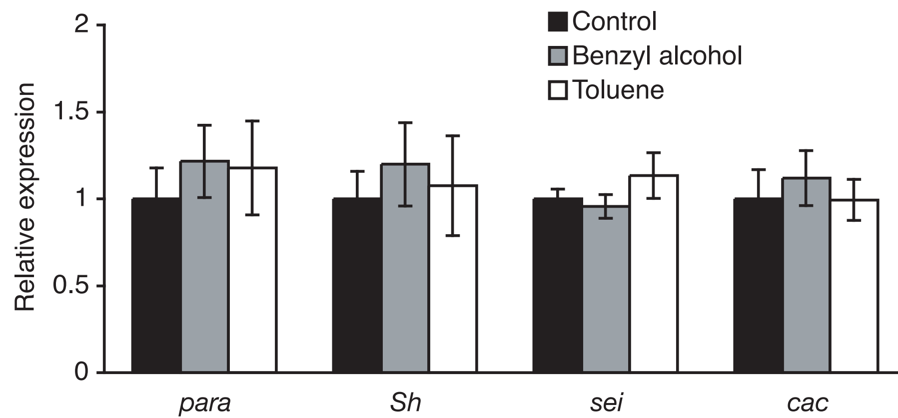

Figure 4.1: Expression patterns of other channels is not affected by solvent exposure. The mRNA abundance of various ion channels was measured relative to *Cyclophilin 1* by real-time RT-PCR from wild type flies that were treated with either 0.4% benzyl alcohol (BA), toluene or were left untreated (Control). The abscissa shows normalized mRNA levels from the each gene relative to control animals that were not exposed to solvents but were otherwise treated identically. Error bars show standard error of the mean.

Mutations in *para*, *Shaker* and *seizure* do not block tolerance to benzyl alcohol

To further explore the role of these ion channels genes in tolerance, mutants in this channels were tested in the benzyl alcohol tolerance assay. The mutants alleles tested were *para*^{63}, *Sh*^{KS133} and *sei*^{1}. Mutants in *cacophony* were not tested since mutants for this ion channels are either lethal or conditional temperature sensitive hypomorphs (partial loss of function).

The *para*^{63} mutation in the voltage-gated Na^{+} channel is also a temperature sensitive allele which becomes paralyzed at elevated temperatures. However, even at room temperature this mutant exhibits significant neurophysiological defects (Huang and Stern, 2002). The *para* gene is located on the X chromosome. In the tolerance test, only male flies containing a single mutated copy of the gene were used. After a single benzyl alcohol exposure, this mutant showed normal tolerance to benzyl alcohol at their second exposure (Figure 4.2A). Additionally, careful observation of the behavior elicited during the exposure to benzyl alcohol revealed that this mutant did not get as hyperactive as wild-type flies at the initial phase of the intoxication with benzyl alcohol and became sedated rapidly.

Similarly, the *Sh*^{KS133} and *sei*^{1} null missense mutations in two different potassium channels in flies (Lichtinghagen et al., 1990; Wang et al., 1997) were not able to block tolerance to benzyl alcohol. Interestingly, contrary to the *para* mutant, both of these mutants became extremely hyperactive as soon as they were exposed to the drug. In addition, it was observed that in the second exposure *Shaker* mutants show a clear reduction in the knock-down time (Figures 4.2B and C).

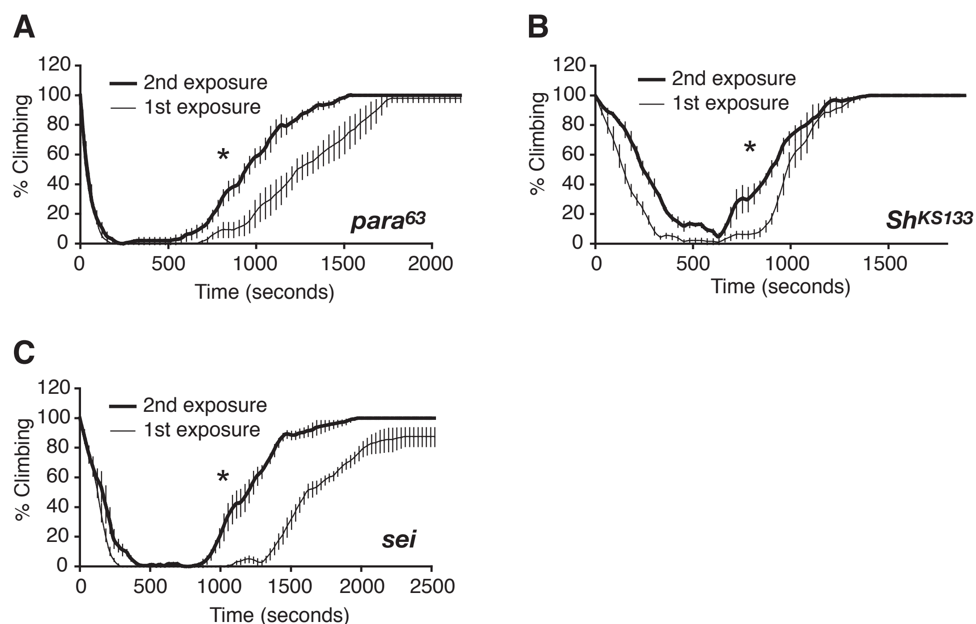

Figure 4.2: Mutants in other ion channel genes show tolerance to benzyl alcohol. Recovery curve of the $para^{63}$ (A), the Sh^{KS133} (B) and sei^{1} mutants (C) after one (thin line) and two (thick line) exposures to 0.3% benzyl alcohol (24 hours between exposures). Values are plotted as a percentage of climbing flies against time at 30 second intervals. An asterisk indicates significant difference from control (n=4, $p < 0.05$). Error bars are standard error of the mean calculated for each data point.

A mutation in *period* blocks tolerance to benzyl alcohol

Previously, I have shown that the acquisition of tolerance to solvent anesthetics is dependent on changes in the expression of the *slowpoke* ion channel gene. This changes appears to be in direct response to changes in the excitability of the nervous system cause by solvent intoxication. Mechanistically, it is thought that activity-dependent regulation of gene expression is carried-out by signal transduction pathways that sense changes in cellular activity and signal to transcription factors that in turn activate or inhibit gene expression. Here, I used mutations in two different genes to independently block two signal transduction pathways that have been shown to be involved in controlling activity-dependent changes in gene expression.

The first mutant tested was dnc^{1}. This mutant eliminates one form of cAMP phosphodiesterase activity (Kauvar, 1982) resulting in increased levels of cAMP (Davis and Kauvar, 1984) and of phosphorylation of the regulatory subunit of a cAMP-dependent kinase (Muller and Spatz, 1989). However, when subjected to the benzyl alcohol tolerance test described previously showed normal acquisition of tolerance (Figure 4.3A) suggesting that changes in cAMP levels do not interfere with tolerance in flies.

The second mutant tested was per^{01}. This mutant eliminates a PAS domain protein that is essential for biological clock functions. The per^{01} flies were subjected to the tolerance test. Figure 4.3B shows that in this mutant, prior benzyl alcohol sedation does not induce drug tolerance.

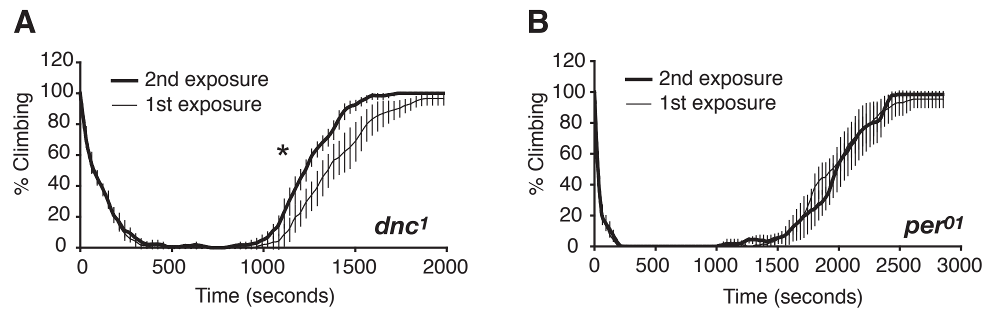

Figure 4.3: Benzyl alcohol tolerance in signal transduction mutants. Recovery curve of the *dnc*^{1} (A) and the *per*^{01} (B) mutants after one (thin line) and two (thick line) exposures to 0.3% benzyl alcohol (24 hours between exposures). Values are plotted as a percentage of climbing flies against time at 30 second intervals. An asterisk indicates significant difference from control (n=4, $p < 0.05$). Error bars are standard error of the mean calculated for each data point.

DISCUSSION

In neurons, electrical activity is established by the combination of different ion channels expressed in these cells. In Drosophila (as in mammals) the repertoire of voltage-gated ion channel genes that a specific cell can express is very large and diverse (Littleton and Ganetzky, 2000). The heterogeneous physiological properties of the different channels gene products allows for appropriate molding of the action potential waveforms characteristic of different neural types but more importantly, provides great flexibility in orchestrating modulation of neural activity in response to sudden changes in excitability (Levitan and Takimoto, 1998).

Surprisingly, of all voltage-gated ion channels tested so far, only the Ca^{+} activated voltage-gated K^{+} channel encoded by the *slowpoke* gene shows changes in expression in response to sedation and hyperactivity induced by volatile solvents. Neither the *Shaker*, *seizure*, *para* or *cacophony* voltage-gated ion channel mRNAs show a significant changes in expression following intoxication by benzyl alcohol or toluene. This suggests that the changes in *slowpoke* are not part of a generalized response that affect transcription from all channel genes. Furthermore, whereas a mutation in *slowpoke* was sufficient to block tolerance, mutations in the other genes tested did not interfere with the ability of flies to acquire tolerance, suggesting a unique role for *slowpoke* in this phenomenon.

It is worth noting however, that the changes in excitability produced in these mutants, may instead affect the sensitivity to the first exposure to benzyl alcohol. It was observed that in the hyperexcitable *seizure* and *Shaker* K^{+} channel mutants, the

hyperactive phase of intoxication was clearly enhanced when compared to wild-type animals whereas the decreased excitability caused by the Na^+ channel mutation *para,* decreased the excitable phase of intoxication. Unfortunately, the methodology used in the tolerance assays fails to report this effects and thus can not be measured in a quantifiable way. These observations were made from visual assessment of behavior and may be unreliable. Perhaps, a more sensitive assay that is able to detect net locomotor activity in flies, and not just their climbing abilities, might give better insight into these effects. This methods are currently been developed in the lab.

Although it is clear that mutations in several of these genes affect the level of sensitivity to specific pharmacological effects of solvent intoxication, changes in their expression patterns are not required for developing tolerance. This data argues that the principle behind tolerance does not necessarily revolve around set levels of sensitivity or resistance towards a specific drug. Rather, tolerance implicates the capacity to produce significant changes in sensitivity in an experience dependent manner. Interestingly, the ion conductance for *slowpoke* channels are up to 10 times larger than all of the channels tested (Wicher et al., 2001). A change in *slowpoke* alone would thus have a greater impact on the excitability of a neuron, making it a reasonable choice for generating large changes in cellular excitability at a lower transcriptional cost.

However, such transcriptional specificity requires precise control of the coupling between changes in activity and changes in gene expression. Furthermore, it is essential that the homeostatic regulations of neural activity do not interfere with the activity of non-homeostatic, Hebbian, activity dependent modifications of neural

circuitry, but instead set the appropriate physiological limits for such changes to occur without resulting in destabilization of neural function (Turrigiano and Nelson, 2000). One hypothesis is that at the transcriptional level, the separation between Hebbian plasticity and homeostatic adaptations might involve independent control of activity-dependent regulation of gene expression through different transcription factors, different signaling pathways or even a spatial separation between the two processes.

The transcription factor CREB has drawn especial attention in the activity-dependent regulation of gene expression since its activation involves many signaling pathways linked to several second messenger systems directly related to changes in membrane depolarization such as changes in intracellular cAMP and/or calcium.

In the cAMP second messenger cascade, accumulation of cAMP in response to activation G-protein-coupled receptors induces activation of CREB by phosphorylation through the cAMP-dependent protein kinase (PKA) (Mayr and Montminy, 2001). Once activated, CREB mediates cellular gene expression by binding as a dimer to a conserved cAMP-responsive element (CRE), TGACGTCA, found in the transcriptional control regions of several CREB target genes. Phosphorylation of CREB also promotes recruitment of the transcriptional co-activator CBP (CREB binding protein) (Mayr and Montminy, 2001).

Gene regulation by the cAMP/CREB signaling pathway has been strongly implicated in Hebbian synaptic plasticity that is associated with long-term memory (Silva et al., 1998; Yin and Tully, 1996; Bourtchuladze et al., 1994; Bartsch et al., 1998). In Drosophila a mutation in *dunce*, a cAMP phosphodiesterase, results in

increased levels of cAMP and of phosphorylation of the regulatory subunit of PKA (Davis and Kauvar, 1984; Muller and Spatz, 1989) and has been shown to interfere with learning and the associated formation of memory (Dudai et al., 1976; Aceves-Pina et al., 1983; Tempel et al., 1983). However, as shown in this study, this mutation does not completely block the acquisition of tolerance to benzyl alcohol, suggesting that tolerance is not dependent on the cAMP cascade.

Intriguingly, the *slowpoke* transcriptional control region contains at least two CRE sequences and shows a coincident up-regulation, after benzyl alcohol sedation, with another CRE regulated gene, *dCrebA*. This gene encodes one member of the CREB family of genes in Drosophila, dCREB-A, and is thought to auto-regulate it self (Rose et al., 1997). Although this evidence points towards a CREB mediated regulation of *slowpoke* after sedation, it seems clear that the activation of this pathway is through a cAMP-independent signal.

Many studies have shown in fact, that CREB can also be activated by cAMP-independent calcium signals. For instance, Pokorska and colleagues (2003) have shown that synaptic activity induces signaling to CREB without increasing global levels of cAMP in hippocampal neurons. In another study, Greenberg and colleagues (1992) showed that in cultured cells, CREB functions as a Ca^{2+} regulated transcription factor and suggested a model whereby activation of voltage sensitive Ca^{2+} channels stimulates activation of a calcium/calmodulin-dependent (CaM) kinase leading to CREB phosphorylation transcriptional activation.

Interestingly, the CREB family member encoded by the Drosophila *dCrebA* contains three CaM Kinase II phosphorylation sites but no PKA phosphorylation sites

(Smolik et al., 1992). This evidence may suggest that the changes in calcium entry associated with sedation by volatile solvents and anesthetics result in the up-regulation of the *slowpoke* gene presumably from the activation of dCREB-A by a CaM kinase signaling pathway.

A different Drosophila cAMP/PKA-activated CREB family member is dCREB-B and is encoded by the *dCrebB-17A* gene. Interestingly, expression from the *dCrebB-17A* gene also generates an alternative spliced product that is a highly conserved homolog of the mammalian cAMP-dependent transcription repressor CREM (cAMP response element modulator) which acts as a specific antagonist of cAMP-inducible transcription (Yin et al., 1995). The *dCrebB-17A* gene may thus be responsible for alternative regulation of CRE mediated genes in a cAMP-dependent manner including those involve in learning and memory (Yin et al., 1994) and perhaps even in the down-regulation of *slowpoke* induced by hyperactivity events (toluene exposure) that lead to sensitization rather than tolerance to sedation.

A more complex scenario is unraveled when one takes a look at the circadian regulation of rest and activity cycles involved in sleep patterns. Rhythmic cycles between arousal and sleep indicate an endogenous component responsible for the regulation of arousal states. Interestingly, when sleeping, animals display very similar behavioral phenotypes to those observed during anesthesia: unconscious states characterized by the lack arousal by auditory, olfactory, somatic and visual stimuli and even electroencephalographic activities that are strikingly similar to those during certain sleep states (Contreras and Steriade, 1996). Studies of sleep regulation can thus offer helpful insights into the mechanisms that result in sedation with drugs. Two

independent studies have shown that sleep in flies share many critical features with mammalian sleep, including prolonged immobility, decreased sensory responsiveness and a even a homeostatic rebound period after rest deprivation (Hendricks et al., 2000; Shaw et al., 2000).

Rhythmic rest and activity cycles in flies are under control of a cell-autonomous molecular clock produced by the transcriptional negative feedback loop of key intracellular proteins. Of these proteins, the two best characterized are encoded by the *period* (*per*) and *timeless* (*tim*) genes (Konopka and Benzer, 1971; Myers et al., 1995). These molecular oscillations are thought to generate changes in locomotor activity and other physiological processes through the rhythmic control of the expression of numerous key output genes (Ceriani et al., 2002; Claridge-Chang et al., 2001).

Expression of *slowpoke* has been shown to cycle in a circadian manner. Furthermore, the slo^4 null mutation (the same one used to block tolerance to benzyl alcohol) results in behavioral arrhythmicity, which implicates *slowpoke* as a central regulator of circadian locomotor activity (Ceriani et al., 2002). Interestingly, as shown here, disruption of the circadian clock by a mutation in the *per* gene, also blocks the *slowpoke* dependent tolerance to benzyl alcohol. This result suggest that the mechanisms mediating circadian regulation of activity may also be involved in the generation of homeostatic responses to changes in activity by many drugs. However, the mechanisms by which clock genes mediate the circadian regulation of *slowpoke,* or the *slowpoke* dependent homeostatic responses remains unclear.

One interesting possibility involves CREB. Experiments conducted in

Drosophila by Belvin and colleagues (1999), using an *in vivo* assay that monitors the expression of a *luciferase* reporter transgene fused to a CRE sequence, have shown that CREB responsive gene expression (of the reporter) has a circadian pattern. However, whether the cycling of the reporter is mediated by dCREB-A or dCREB/ CREM-B or if it is cAMP- or Ca^{2+}-dependent remains unclear. Belvin *et al*. (1999) showed that the circadian patterns of the CRE-*luciferase* reporter gene expression were affected by mutations in *dCrebB* and in genes involved in the cAMP pathway, but were not completely abolished. This suggest that circadian activation of CRE-mediated expression might involve more than one signal. Interestingly, other studies have shown that in mammals, activity-dependent cytoplasmic calcium levels and NMDA-evoked calcium currents in the suprachiasmatic nucleus oscillate in a circadian manner (Colwell, 2000; Colwell, 2001). This data suggest that circadian modulation of behavioral activity may be mediated through CREB by both calcium and cAMP signals.

In order to effectively mediate cycling of high and low levels of expression from clock output genes within one circadian period, it seem fairly reasonable to expect that a CRE-mediated circadian expression involves both activation and repression of transcription that alternate through the day. If so, then this can be achieved by the activation of transcription through Ca^{2+}-dependent dCREB-A activation and repression through cAMP-dependent dCREB-B activation.

Additional speculations about the mechanisms by which clock genes mediate changes in arousal states through the circadian regulation of *slowpoke,* or *slowpoke* dependent homeostatic responses can be made from experiments performed on the

sleep rebound phenomenon.

In two independent studies (Hendricks et al., 2000; Shaw et al., 2000) it was shown that as in mammals, flies exhibit a homeostatic rebound period after rest deprivation. In these studies scientist have shown that a fly's resting period after stimuli driven rest deprivation is significantly increased but the timing of the rest–activity cycle was not altered. Suggesting a homeostatic mechanism that mimics that of cross-sensitization to sedation after exposure to hyperactivity inducing solvents (i.e. toluene, Figure 3.4C). In this analogy, the sleep depriving stimulus (mechanical) resembles the hyperactive state induced by toluene pre-exposure while the rebound prolongation of sleep resembles the increased duration of sedation by benzyl alcohol.

Interestingly, Hendricks and colleagues (2000) also showed that the homeostatic sleep rebound response was blocked in *tim* mutant flies but remained normal in *per* mutant flies. Furthermore, a transgene carrying *tim* under control of the *tim* promoter, rescued the rest rebound in the *tim* mutant. These results led the authors to conclude that even thought together *per* and *tim* are essential for circadian function, the role of the two genes in rest homeostasis might be different and independent from the circadian clock (as *per* mutants are arrhythmic, and still show significant rest rebound). Clearly, *tim* has a function in rest homeostasis that goes beyond its central clock function. In contrast, I have shown that a mutation in *per* blocks tolerance to sedation by benzyl alcohol, suggesting a role for *per* that goes beyond its central clock function.

Although there are many interpretations to this result, one interesting hypothesis is that the homeostatic mechanisms that mediate the responses to

hyperactivity (sleep deprivation) and to underactivity (sedation by anesthetics) are mediated by two different clock genes. Perhaps, *tim* alone somehow mediates repression of CRE-responsive genes (*i.e. slowpoke*) through cAMP-dependent activation of the repressor form of dCREB-B (CREM) leading to decreased of arousal states (prolonged sleep). While *per* alone somehow mediates the activation of CRE-responsive genes (*i.e. slowpoke*) through Ca^{2+}-dependent activation of dCREB-A resulting in higher arousal states (tolerance to sedation).

Unfortunately, whether the clock genes independently modulate *slowpoke* expression in opposite ways is not know. Neither it is known if different second messenger signals mediate opposing CREB effects in *slowpoke*. But the experiments discussed here clearly involve a very complex mechanism by which the two clock components acting as a molecular complex mediate the natural variations in behavioral activity, and when acting independently mediate homeostatic responses to opposing stimuli. This hypothesis opens new exciting questions that remain to be answered.

METHODS

Fly stocks

All flies used in the experiments discussed in this chapter were raised on standard cornmeal/molasses/agar medium. Flies were kept in a room with constant temperature (22 ºC) and 12:12 hrs light:dark cycles. Flies that emerged from pupae were collected over a 2-day period, transferred to fresh food, and studied between three to five days later. In this way, all flies are roughly between five to seven days old. Genotype of mutant stocks were Sh^{KS133}, sei^{ts1}, dnc^{1}, per^{01} and $para^{63}/x\text{^}x$.

Benzyl alcohol tolerance assay

Benzyl alcohol tolerance was determined as described earlier (Chapter 3, Methods) with a few modifications. In the first exposure, flies were treated in replicates of four with benzyl alcohol (0.3%) or vehicle (100% acetone – evaporated to dryness) as described in Chapter 2. Twenty-four hours later, treated and control flies were simultaneously exposed to benzyl alcohol until all flies were sedated (approximately 10 minutes). At this point flies were transferred to a solvent free glass vial for recovery. Snapshots were taken every 30 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 four tubes) against time at 30-second intervals. The data and statistics shown in the figures are derived from a protocol in which each assay is performed four times. 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 (Hosmer et al., 2002). However, in behavioral assay we have also included error bars that represent the standard error of the mean for each individual data point.

Quantitative real-time RT-PCR

Total RNA was extracted from 25 wild type flies 6 h after the start of the treatment by using a single-step RNA isolation tissue protocol as in Chapter 2. The mRNA expression from the *para, seizure, cacophony* and *Shaker* genes was determined by quantifying abundance relative to *Cyclophilin 1* (*Cyp1*) using the SYBR Green I nucleic acid stain real-time RT-PCR assay as in Chapter 2. The primers used were:

- *para*: 5' -ggtgctgcgagcgcttaa- 3' and 5' -ggataatcacatcgcgcagat- 3'.
- *seizure*: 5' -tggtgatgtacacggccatt- 3' and 5' -atgacaattggatcggagttgat- 3'.
- *Shaker*: 5' -gcggattaaggtttgagacacaa- 3' and 5' -gtaccgtaatctccgagctggat- 3'.
- *Cacophony*: 5' -tagtgaaggagggcgaatcaga- 3' and 5' -gcttgtggtgttattgcatacgaa- 3'.
- *Cyclophilin* 1: 5' -accaaccacaacggcactg- 3’ and 5’ -tgcttcagctcgaagttctcatc- 3’.

Chapter 5: Electrophysiological Effects of *slowpoke* Modulation

INTRODUCTION

The studies presented in the previous chapters have demonstrated that an increase in the expression of the gene *slowpoke* is both sufficient and necessary for the development of tolerance to sedation from anesthetics. As discussed previously, this evidence suggests that the molecular response and the long-term behavioral consequences of sedation manifested in the form of rapid drug tolerance are cause and effect of a homeostatic mechanism that modulates neuronal excitability (Ghezzi et al., 2004). One hypothesis is that the increased BK channel expression from the *slowpoke* gene enhances net neural excitability and thereby helps the nervous system to resist sedation.

Although it is generally expected that increased K^+ channel activity would cause hyperpolarization of the cell and reduce neural excitability (Sun and Dale, 1998; Orio et al., 2002; Gribkoff et al., 2001) there is also substantial evidence that increased *slowpoke* channel activity can induce an opposite effect on neural excitability of certain circuits. It has been proposed that a fast re-polarization of membrane potentials by the *slowpoke* channel will prevent the prolonged inactivation of voltage-gated calcium channels that mediate calcium dependent transmitter release, thus reducing the refractory period of the channel and allowing an increase in repetitive firing (Warbington et al., 1996; Lovell and McCobb, 2001; Pattillo et al., 2001).

However, the long term effects of the up-regulation of the *slowpoke* gene on

the excitability of a neural circuit in Drosophila remain unknown. It is also unclear whether the effects will be in any way similar to the ones elicited by sedation so as to explain the role of *slowpoke* in tolerance to drugs. In this chapter, I explore the long-term effects of sedation and the up-regulation of *slowpoke* on the excitability of the giant fiber pathway, a very well characterized neural pathway involved in the visual escape response in Drosophila and that is well suited to answer this particular question.

The visual escape response is the fly's instinctive reflex to escape after sudden changes in light intensity. The escape response can be elicited experimentally by electrical stimulation across the fly's brain. This stimulus is thought to activate a pair of interneurons called the giant fibers that descend from the brain into the thoracic central nervous system and subsequently activate several muscles involved in jumping and flying and mediate the escape response. The response can be accurately monitored by recording from these muscles (Wyman et al., 1984).

Previous studies have shown that during sedation by anesthetics, electrical induction of this pathway is significantly inhibited in a dose dependent manner (Lin and Nash, 1996). Therefore, this preparation offers an invaluable tool to directly study the long term physiological effects of sedation-induced *slowpoke* expression over a simple but relevant neuronal circuit.

RESULTS

The giant fiber pathway in Drosophila

In the giant fiber pathway, the giant fiber conducts action potentials from the brain, through the cervical connective that links the head to the thorax. In the thorax, the giant fiber synapses with an inter-neuron (electrical synapse) which connects to a motor neuron that terminates on the dorsal longitudinal (DLM) flight muscles (Wyman et al., 1984). Electrical stimulation of the giant fiber circuit by electrodes placed on the eye elicit, after a short delay, evoked potentials in the DLM that can be recorded by an electrode placed dorsally in the thorax (Figure 5.1A). As the intensity of the stimulation increases, the latency (delay) of the response undergoes distinct shifts in duration. At relatively low stimulating voltages (10-16 volts) the response in the muscle appears with a latency of approximately 4 ms. This response is usually called the long latency (LL) response (Elkins and Ganetzky, 1990; Engel and Wu, 1996; Lin and Nash, 1996). At higher voltages (18+ volts) the latency drops to a surprisingly constant 1.4 ms, and is referred as the short latency (SL) response. In some cases an intermediate latency response (IL) of approximately 2 ms can also be detected (Figure 5.1B).

It has being suggested (Levin and Tracey, 1973; Tanouye and Wyman, 1980), that with small voltages, stimulation of the brain will trigger the activation of afferent neurons located pre-synaptically to the giant fiber. Thus, the long latency response will result from the subsequent activation of the giant fiber. On the other hand, higher voltages that propagate across a larger area of the brain, will directly activate the giant fiber, resulting in a shorter latency (SL).

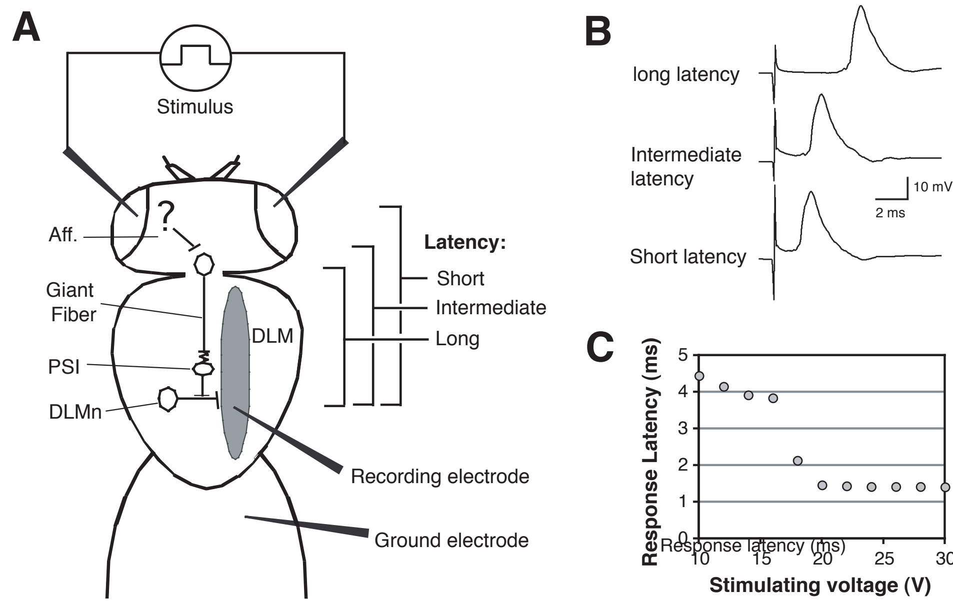

Figure 5.1: The giant fiber pathway. A) In the giant fiber pathway, the giant fiber, which receives inputs from visual or other neural afferent pathways (aff.) is electrically connected to the peripheral synapsing interneuron (PSI) which activates the DLM motoneurons (DLMmn). The DLMmn synapses upon the dorso-longitundinal flight muscle (DLM). Stimulating potentials, passed through electrodes on the eyes, activate neural afferent pathways (aff.) that trigger a response that can be recorded by an electrode in the DLM. B) Different stimulating voltages will trigger the activation of the pathway at different points, resulting in responses with different latencies that can be divided in three main categories: long, intermediate and short. Traces show typical long, intermediate and short latency responses evoked by brain stimulation. C) Response latencies from a single fly plotted as a function of increasing stimulating voltage. Increasing the stimulus voltage decreases the latency of the evoke response. The three different groups can be clearly distinguished. The larger potentials are thought to directly trigger activation of the giant fiber and have a very constant short latency of approximately 1.4 ms.

The following frequency of the giant fiber pathway

In order to accurately measure the excitability of a neural circuit involved in the giant fiber pathway I have chosen to measure the ability of the system to follow high frequency stimulation. This type of stimulation offers a reliable way to measure the refractory period of the circuit (Lin and Nash, 1996; Engel and Wu, 1992). For this, trains of stimuli at ever increasing frequency were delivered to determine the following frequency of the response from the giant fiber pathway.

Although the following frequencies of both the short and long latency responses can be tested in this way, the long latency response is significantly more sensitive to high frequency stimulation than the short latency response as well as more variable. In fact, as shown in Figure 5.1C, individual long latency responses actually include a wide range of different latencies that change slightly with small changes in the stimulating voltages. This may be an indicative of a complex set of unknown afferent pathways that trigger the activation of the giant fiber and that will be activated differently by small changes in electrode placement and thus resulting in recordings with high variability between flies. On the contrary, the short-latency response is extremely constant and is stable in response to high frequency stimuli (Tanouye and Wyman, 1980) offering a more precise way to detect small changes in excitability like the one expected to happen after *slowpoke* induction. The short latency response was thus chosen for determining the following frequency in all subsequent experiments.

Three trains of ten stimuli were delivered and the number of failures counted. A failure is the absence of an action potential in the muscle in response to an evoked

stimulation. The frequency of the stimulus trains was gradually increased and as the refractory period was exceeded the neurons failed to fire. The animals were allowed to rest between each stimulus train. In this manner, we identified a stimulation frequency that was too high for the pathway to follow. Then the last successful frequency was re-tested to confirm that the giant fiber pathway was not exhausted. The following frequency with 50% response (FF50) was also determined by linear interpolation (Engel and Wu, 1992). Since the FF50 was calculated from the linear phase of the frequency response curve for each fly, it is an independent measure of the response rates compared to that calculated from the frequency response curves.

It was reasoned that a decrease in the refractory period would lead to a increase in the following frequency of the neural circuit. If increased *slowpoke* expression enhanced neural excitability by reducing the refractory period, then we should observe a higher following frequency in animals in which expression of the *slowpoke* gene was induced.

In order for this method to produce an accurate report of the following frequency of the neurons in the giant fiber pathway, the following frequency of the flight muscle must substantially exceed that of neurons. In figure 5.2, I compared the following frequency of the giant fiber pathway and flight muscle directly stimulated with an extracellular electrode. As expected, low frequency stimulation of the short-latency pathway elicits responses in the muscle for each of the 30 stimuli. However at stimulation frequencies higher than 80 Hz, the circuit becomes refractory and no response is detected from the DLM muscle. This muscle however, when stimulated directly at the thoracic ganglion, will fire successfully even after stimulating

frequencies higher than 240 Hz suggesting that the failures seen after the giant fiber stimulations are neuronal rather than muscular in nature.

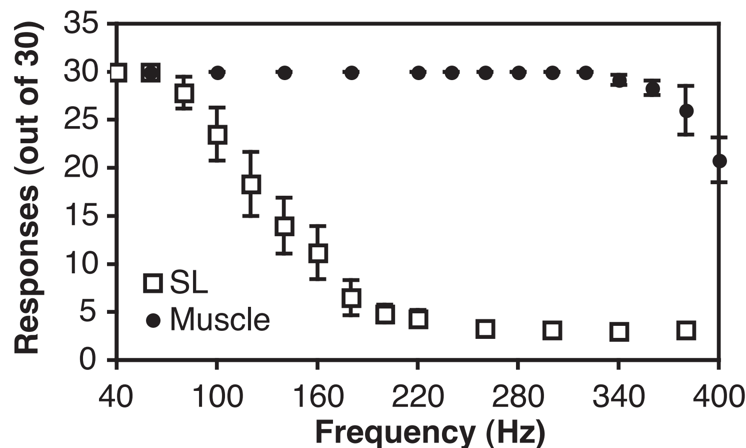

Figure 5.2: Frequency characteristics of the S-L and direct muscle stimulation. The following frequency of the short-latency response of the giant fiber pathway was determined. Three trains of ten stimuli were delivered through the eyes at different frequencies and actions potentials recorded at the end of the giant fiber pathway in the DLM (SL, open boxes). Responses (out of 30 possible) at each frequency were counted and plotted against frequency. Closed circles are DLM action potentials produced by direct-muscle stimulation via electrodes in the thoracic ganglion. Note that the capacity of the DLM to follow high frequency stimulations far exceeds that of the giant fiber pathway. Error bars are SEM, n=5.

Sedation enhances excitability of the giant fiber pathway

To test if sedation causes long term effects on the excitability of the giant fiber pathway, the following frequency of the short latency response of flies that have being previously sedated with benzyl alcohol was determined.

A single benzyl alcohol sedation, twenty-four hours prior, substantially increases *slowpoke* expression (chapter 2). Figure 5.3 shows that it also increases the following frequency of the giant fiber pathway. A significant rightward shift in the frequency response curve is observed (Figure 5.3A) with an average increase in the FF50 of 32 hertz (27%) over the non treated control (Figure 5.3B). To test whether the loss of *slowpoke* expression, which prevents the acquisition of sedation-induced tolerance, also blocks this electrophysiological phenotype, the effects of sedation in the following frequency of a *slowpoke* mutant was tested. However, elimination of *slowpoke* expression in muscles would cause a broadening in the evoked potentials recorded from the DLM (Brenner et al., 2000) and interfere with this assay. Previously, It has been shown that the *ash*2^{18}/*slo*^{4} heteroallelic combination can be used to eliminate *slowpoke* expression from neurons but not from muscles. The *ash*2^{18} mutation is a small, recessive lethal deletion which removes the neighboring gene and also the transcriptional promoters that drive *slowpoke* in neurons. Fortunately, the muscle promoter is intact and functional. The intrinsic electrophysiological properties of flight muscle in *ash*2^{18}/*slo*^{4} transheterozygotes appears normal (Atkinson et al., 2000). Figure 5.3C and 5.3D show that prior sedation fails to produce an increase in following frequency in the *ash*2^{18}/*slo*^{4} animals, indicating that genetic ablation of *slowpoke* expression in the nervous system also blocks this sedation-induced

phenotype.

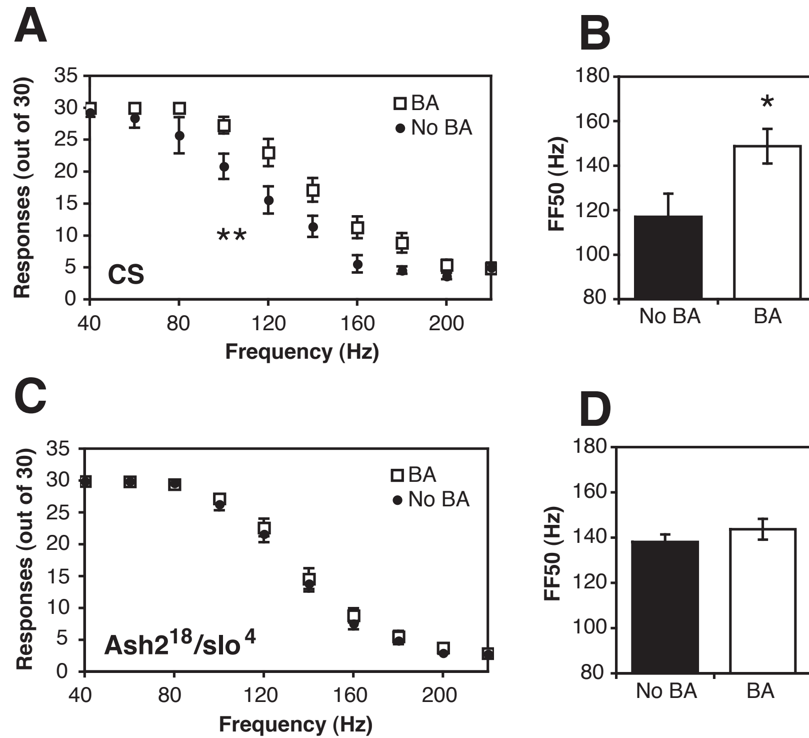

Figure 5.3: Sedation enhances the following frequency of the neural visual escape pathway and is dependent on a functional *slowpoke* gene. Sets of three trains of ten stimuli where delivered at different test frequencies through large voltage stimulation on the eyes (SL) to animals that were sedated with benzyl alcohol for 30 minutes 24 hours earlier (open boxes) or age-matched controls that were not sedated (closed circles). The number of responses (out of a total of 30 possible) at each frequency were counted and plotted against frequency (A and C). Additionally, the following frequency with 50% response (FF50) for each animal was calculated by interpolation (B and D). A-B) Previously sedated wild type CS flies which have a functional *slowpoke* gene show a significant rightward shift in the response curve in comparison to the non-heat shocked control (n=7, ** indicate $p<0.02$ repeated measures ANOVA) and showed an increase in the FF50 of the giant fiber pathway (* indicates $p<0.05$, Student's t-test). C-D) Ash2^{18}/*slo*^{4} transheterozygotes do not express *slowpoke* in the nervous system and sedation did not affect the frequency response curve nor the FF50 of their giant fiber pathway.

Induction of *slowpoke* enhances excitability of the giant fiber pathway

In order to test if the *slowpoke* induction by itself was sufficient to phenocopy the electrophysiological effect of sedation on the following frequency of the giant fiber, a transgenic fly carrying an inducible copy of a *slowpoke* cDNA was used. With this transgene one can temporally control the time of induction by giving a brief heat-shock to the flies which resembles a benzyl alcohol treatment both in duration and in timing of the event, 24 hours prior to testing in this case. This form of induction has been shown to produce levels of *slowpoke* that mimic those induced by benzyl alcohol as well as confer increased resistance to the drug compared to non heat-shocked control in a way that resembles tolerance to the drug (Ghezzi et al., 2004).

A single heat pulse caused B52 flies to show an increase in neuronal following frequency with a shift in the response curve slightly to the right (Figure 5.4A) and an average increase in the FF50 of 21 hertz or 15% over the nontreated control (Figure 5.4B). However, when non-transgenic control animals are subjected to the same heat pulse they do not show an increase in following frequency (Figures 5.4C and 5.4D). In fact, these flies show a non-significant but notable decrease in the number of successful responses after electrical stimulation compared to non heat-shock controls. This result is not surprising since it was previously shown that heat-shock by itself causes a state of hyperactivity which results in a significant decrease in *slowpoke* expression (Chapter 2).

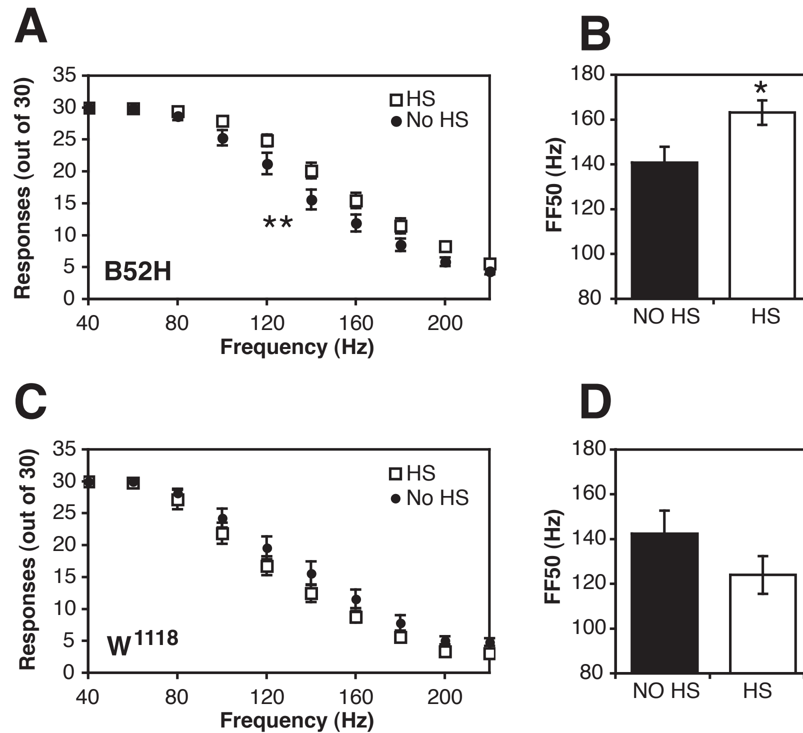

Figure 5.4: Transgenic induction of *slowpoke* expression increases the following frequency. Sets of three trains of ten stimuli where delivered at different test frequencies through stimulation on the eyes (SL) to animals that were heat-shocked (37°C) for 30 minutes 24 hours earlier (open boxes) or age-matched controls that were not heat-shocked (closed circles). The number of responses (out of a total of 30 possible) at each frequency were counted and plotted against frequency (A and C). Additionally, the following frequency with 50% response (FF50) for each animal was calculated by interpolation (B and D). A-B) In B52H flies, heat shock induction of the B52H transgene shifts the response curve slightly to the right in comparison to the non-heat shocked control (** indicate $p<0.01$, n=27 HS, 22 No HS, repeated measures ANOVA) and induces a significant increase in the FF50 (* indicate $p<0.02$, Student's t-test). C-D) The same heat shock protocol does not produce a significant difference in the parental non-transgenic stock. (n=13 HS, 12 NO HS).

Discussion

The strong link between an increase in the expression of the *slowpoke* gene and the development of tolerance to sedation suggests that at a functional level, the up-regulation of *slowpoke* is part of homeostatic response that enhances net neural excitability and thereby helps the nervous system to resist sedation and leads to drug tolerance in flies. Data presented here is consistent with the idea that increased neural expression of *slowpoke*, is in some respect, a neural excitant. This is an unusual role to postulate for a K^+ channel. Certainly, in some preparations, increased BK channel activity clearly reduces neural excitability (Sun and Dale, 1998; Orio et al., 2002; Gribkoff et al., 2001). However, in other preparations, BK channel activity has been positively correlated with neural excitability (Warbington et al., 1996; Lovell and McCobb, 2001; Van Goor et al., 2001; Pattillo et al., 2001). It has been proposed that the increase in BK channel activity limits the instantaneous response of the cell, it augments the capacity for repetitive neural activity by reducing the neural refractory period (Warbington et al., 1996; Lovell and McCobb, 2001). The refractory period is the period of time that must elapse before the neuron can fire again.

Drosophila is a difficult organism in which to do central nervous system electrophysiology. However, the giant fiber pathway is well suited for this particular question. The giant fiber synapses with an interneuron that connects to a motor neuron which triggers a response from a muscle fiber. Thus, muscle recordings report the firing pattern of the giant fiber pathway. When stimulated with low potential used to activate the forebrain, a so-called long-latency response can be triggered. In these case, the remainder of the brain actively propagates the signal before it activates the

giant fiber. However, by using a higher stimulation potential one can, in essence, stimulate the entire brain and directly activate the giant fiber. This results in what is called the short-latency response. Direct stimulation of the giant fiber is easily confirmed by noting the latency of the response. In this study, the use of the short-latency response was preferred since it provides a better defined CNS circuit. The refractory period of these neurons determines the capacity of the giant fiber pathway for repetitive activity.

Prior sedation of flies increases the following frequency of the short-latency response of the giant fiber pathway. This enhancement is *slowpoke* dependent in that mutations in *slowpoke* block it. Most importantly, it can also be produced by the induction of *slowpoke* expression from a transgene. This unambiguously indicates that an increase in *slowpoke* neural expression is sufficient to reduce the refractory period of the pathway.

Although the enhancement in following frequency caused by the transgene is less than that caused by sedation. In general, the transgene produces higher levels of *slowpoke* expression than that produced by sedation of wild type flies (Ghezzi et al., 2004). This might indicate that sedation also enhances following frequency by a secondary means that is independent of the *slowpoke* gene. Alternatively, sedation might produce more expression in the giant fiber pathway than does induction of the transgene. Unfortunately, all of the measurements of *slowpoke* expression are from the nervous system in general. It is not yet possible for us to measure expression levels specifically in the giant fiber pathway.

It is expected that an acquired enhancement in repetitive firing, as the one

induced by prior sedation and/or *slowpoke* up-regulation, results in a decrease in the immediate effects of sedation causing increased resistance. Interestingly, experiments by Lin and Nash (1996) have demonstrated that the giant fiber pathway is sensitive to sedation by general volatile anesthetics. Specifically, it was reported that the following frequencies of the long and short latencies drop significantly during anesthesia. However, it was also noted that the sensitivity to anesthetics of the two responses differed substantially. The long latency response was potently inhibited at concentrations which are comparable to those needed to interfere with postural control, whereas the short latency was only inhibited at much higher doses.

It has being proposed, that the difference in sensitivity may be revealing of neural components in the brain that are more sensitive to anesthetics than those down stream. However, this may very well be due to the increased complexity of the circuit involved in the long latency response. Indeed, most behavioral and physiological indicators of sedation at clinically-relevant doses of anesthetics such as complex coordination of sensory and motor systems implicate more complex neuronal assemblies than the giant fiber circuit involved in the short latency response and therefore it is not surprising that these physiological indicators are more susceptible to anesthetics.

One can argue that a neural circuit with a higher number of synapses is more liable to fail or be disrupted. In fact, even in the absence of anesthetics, the long latency response is considerably more delicate than the short latency response, with following frequencies as low as 2 hertz (Lin and Nash, 1996). On the contrary, the short latency response, involves only three neural connections (one electrical synapse,

one chemical synapse and one neuromuscular junction) and can follows stimuli in excess of 100 hertz.

Although, functional tolerance to any drug is likely to involve a great many neural components and in certainly other genes have been implicated in the production of tolerance to drugs such as ethanol (Wen et al., 2005; Berger et al., 2004; Dzitoyeva et al., 2003; Park et al., 2000), the *slowpoke* gene is uniquely positioned to be a homeostatic regulator of neural excitability. The encoded channel has one of the highest conductance of any neural ion channel, thus small changes in its density, caused by its transcriptional response to sedation, can have a large influence on membrane excitability. In conclusion, it is proposed that sedation causes changes in the *slowpoke* expression causes a significant increase in the excitability of the nervous system that leads to drug tolerance in flies.

METHODS

Fly stocks

Drosophila stocks used in this study were Canton S (wild type), B52H, w^{1118}, slo^4 and the $ash2^{18}/slo^4$ trans-heterozygote. 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 (Atkinson et al., 1998). The B52 transgene is constitutively active but is substantially induced by a brief heat shock. Flies were raised on standard cornmeal/ molasses/agar medium at room temperature (25°C) in a room with circadian control of illumination (12 light, 12 hours dark). Flies that emerged from pupae were collected over a 24 hour period, transferred to fresh food, and studied three to four days later.

Benzyl alcohol treatment and *slowpoke* induction

Benzyl alcohol exposure was performed by coating 30 ml glass vials with 200 ul of a solution of benzyl alcohol in acetone (0.4%). These experiments included an acetone control. The vials were continuously rotated for 45 minutes at 22°C to evaporate the acetone, leaving a thin coat of evenly distributed benzyl alcohol. Ten to fifteen age-matched female flies were placed in each vial and exposed to the benzyl alcohol until sedation (10 to 15 minutes).

Expression of *slowpoke* was induced from a HSP70:slo transgene in the B52H flies by placing them in a 37°C humidified incubator for 30 minutes. Mock induction

was performed with w^{1118} flies in the same way.

Fly set-up for electrophysiology

Individual adult female flies were placed in a small centrifuge tube and briefly anesthetized by placing the tube in ice for exactly 3 minutes. The sedated fly is dropped over a small mount of bee's wax on a microscope slide. Using fine forceps, the fly is fixed in place before it wakes up by embedding it's legs in the wax so that the fly has a natural standing position (horizontal - dorsal side up) but is unable to walk or fly away (Figure 5.5A). The head and thorax are glued together to prevent movement by application of small amounts of superglue using a pulled thin capillary tube. After drying for three minutes, small drops of conducting gel (Signa Creme, Parker Laboratories, Inc., Fairfield, NJ) were applied to the eyes using a syringe with a gauge 27.5 needle (Figure 5.5B). For experiments that involve treatments with anesthetics or heat-shock, flies were treated 24 hrs prior to the recordings and returned to food vials for recovery. Both, benzyl alcohol and heat-shock treatments were performed at mid day (6th hour of light).

The fixed fly was transferred to a 3X magnification stereoscope stage that is set over a air table. Electrodes were inserted in the fly using micromanipulators (Narishige, Tokio, Japan) that were also set over the air table. All electrodes were insulated tungsten wires (FHC Inc, Bowdoinham, ME).

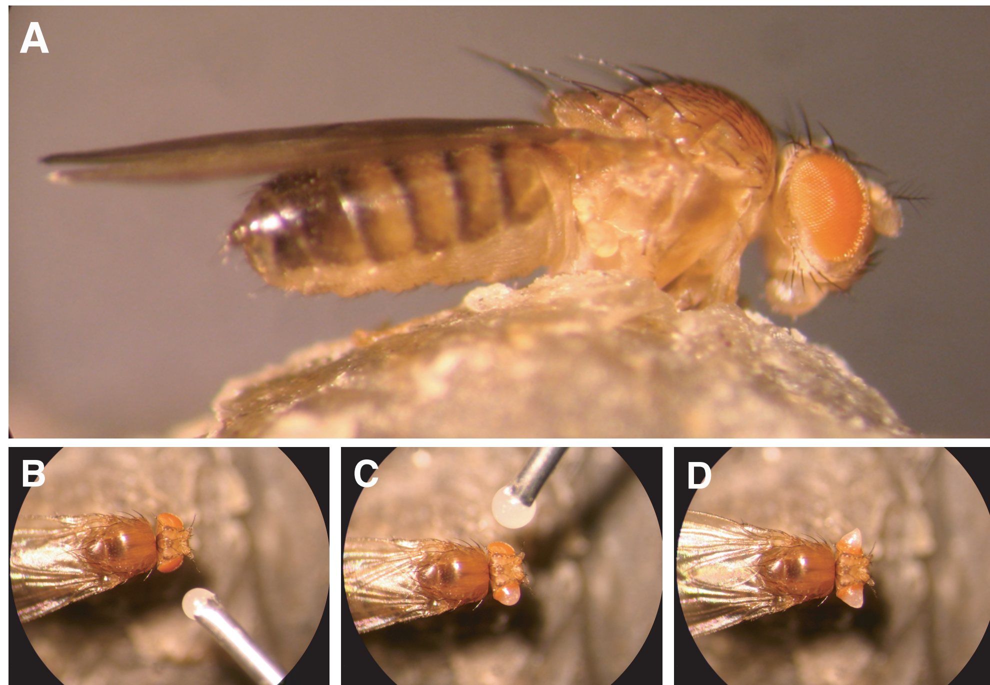

Figure 5.5: Fly set-up for electrophysiological stimulation and recording. A) Picture of a fly fixed in a wax mount. Legs are embedding in the wax so that the fly has a natural standing position (horizontal - dorsal side up) but is unable to walk or fly away. B, C, D) Sequential pictures of a fly during the application of small drops of conducting cream using a syringe with a gauge 27.5 needle.

A 200 um diameter stimulating electrode was placed into the conductive gel on each eye at an angle of approximately 45 degrees to the anterior posterior axis of the fly so that it is perpendicular to the tangent of the center of the eye (Figure 5.6). When inserted, the electrode tips are immersed in the conducting cream covering the eyes and make contact with the surface of the eye, but do not pierce the eye. A 75 um diameter recording electrode was inserted coming dorsally, parallel to the anterior-posterior axis of the fly (when viewed from the top) but at a 45 degree angle above the plane of the microscope stage (Figure 5.6). The electrode is inserted through the cuticle slightly to the right of the center of the line that divides the notum and the scutellum into the right-uppermost dorsolongitudinal muscles (DLM) that lies just beneath the cuticle. A 200 um diameter reference electrode was inserted into the abdomen coming at 45 degrees above the plane of the microscope stage and 45 degrees left of the anterior-posterior axis.

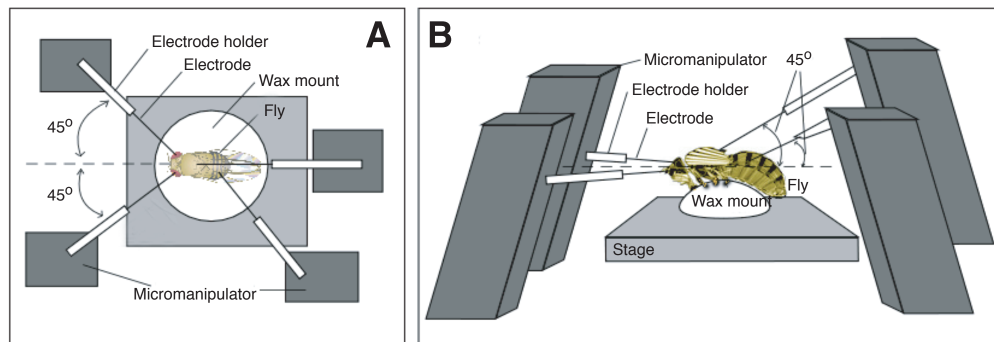

Figure 5.6: Set-up of the electrophysiology stage. A fly that has been fixed on a wax mount is placed on a stereoscope stage. Electrodes are positioned in the fly at the angles shown using micromanipulators A) Top view of the recording stage. B) Side view of the recording stage.

Electrophysiology

Stimulating potentials of 0.1 ms duration were generated using a S48 square pulse stimulator, isolated with a SIU5 Stimulus Isolation Unit (Grass-Telefactor, West Warwick, RI). Responses from the DLM muscles were amplified with a Microelectrode Amplifier Model 1800 (A-M systems, Inc., Carlsborg, WA), digitized by a DigiData 1200 (Axon instruments, Sunnyvale, CA) and recorded for analysis on a PC using FETCHEX, pCLAMP 6 software (Axon instruments, Sunnyvale, CA).

For determining latencies, a stepping protocol was employed. In this protocol a 10 volt stimulus, 0.1 ms in duration, is delivered and the muscle response recorded. The voltage is then increased by two volts and another 0.1 ms stimulus is delivered. The process is repeated until the stimulating voltage reaches 30 volts. Latencies of the responses from each stimulus are measured using FETCHEX, pCLAMP 6 software (Axon instruments, Sunnyvale, CA) from the beginning of the stimulus artifact to the beginning of the evoked muscle response. Results are represented as a voltage/ latency plot for each fly tested.

For high frequency stimulation, single stimuli, 0.1 ms in duration, of increasing amplitude were first delivered to the eyes until a response with a constant short latency of approximately 1.4 ms was detected. A short latency response is indicative of direct stimulation of the giant fiber pathway. While determining the following frequency (see below), the stimulation potential was four volts over the short latency threshold in order to insure that the giant fiber pathway was always directly activated. For direct muscle stimulation, two stimulating electrodes were

inserted laterally (one from each side of the fly) into the thoracic-abdominal ganglion and stimulated at 5 volts.

Following frequency was determined with a stimulus paradigm that consisted of three trains of ten stimuli at each test frequency, with five seconds between trains. The stimulus frequency was increased by 20 Hz after each three train test, starting at 40 Hz up to 220 Hz for short latency stimulation and up to 400 Hz for direct muscle stimulation. The number of stimulus responses detected in the muscle (out of 30) were counted for each frequency. Averaged responses were plotted against frequency to generate a frequency response curve. Two-way repeated measures ANOVA was used to determine significance, although the error bars in the figures are SEM for each point. All recordings were performed at the same time of day (around the sixth hour of light according to the flies circadian lighting).

For each fly, following frequency with 50% response (FF50) was independently calculated from the linear phase of the frequency response curves by linear interpolation of the frequency that elicited 15 responses (Engel and Wu, 1992). Significance was determined by Students t-test.

Chapter 6: Summary and Conclusion

The ability of the nervous system to generate carefully coordinated movements, perform complex computational tasks or exercise precise control over several physiological functions, relies on an exquisitely controlled balance between excitatory and inhibitory signals within and between nerve cells. The perturbation of this balance, will often result in the aberrant functioning of these processes. Many volatile solvents can produce significant alterations in the activity of the nervous system causing severe physiological and behavioral effects that range from uncontrolled hyperactivity to profound sedation. These attributes have led to the use of organic solvents as valuable clinical anesthetics in some cases or as detrimental drugs of abuse in other unfortunate cases.

Upon alteration of neural activity by these drugs, the nervous system triggers homeostatic mechanisms which ensure that the activity returns to reasonable physiological limits, thus preventing individual neurons from firing excessively or become completely silent. These changes can occur as either fast and transient responses that oppose the immediate effects of the perturbations or as slow and long-term adaptive processes which prepares the system for future perturbations. In the case of drug induced alterations of activity, homeostatic long-term neural adaptations are often manifested in the form of tolerance. In this study, tolerance was defined as an acquired decrease in sensitivity to the effect of a drug that results from prior exposure to that drug.

Since the excitability properties of neurons are determined by the types, distribution, and density of ion channels they express, changes in the expression of channels genes can affect cell resting potentials, action potential threshold and duration, repetitive firing, and neurotransmitter release. Here, it was hypothesized that modulation of ion channel genes may represent a homeostatic mechanism that underlies tolerance to drugs. Using a Drosophila model system to investigate the molecular mechanism behind this phenomenon I have shown that changes in the expression of the calcium-activated, voltage-gated potassium channel gene *slowpoke* result in changes in the excitability of the nervous system and leads to tolerance to sedation.

First we have seen that exposure of fruit flies to solvent anesthetics results in a brief initial phase of exaggerated locomotor activity followed by a long period of deep sedation, effects that remarkably parallel those observed in higher organisms including humans. Interestingly, exposure to these drugs also results in the modulation of expression of the *slowpoke* gene. In these studies it was shown that the induction of *slowpoke* expression by a drug is closely linked to the level of activity induced by the drug. Drugs or doses that induce sedation also induce *slowpoke* expression, while drugs or doses that induce hyperactivity cause a decrease in *slowpoke* expression. This data suggests that the transcriptional regulation of *slowpoke* is under control of activity-dependent mechanisms that can monitor and respond to changes in activity induced by the drug rather than to the presence of the actual drug.

Second, flies as well as humans, may develop tolerance to more than one

effect of these drugs. One particular effect that was extensively studied here, is the duration of sedation induced by these drugs. After a single exposure to benzyl alcohol flies will recover faster from the sedative phase of a second intoxication by the same drug. Interestingly, the development of tolerance to sedation in flies is dependent on *slowpoke* expression. A mutation that eliminates *slowpoke* expression in all tissues or specifically in the nervous system of Drosophila, also eliminates the appearance tolerance, while artificial induction of *slowpoke* expression using an inducible transgene will cause resistance to sedation in naïve flies.

Furthermore, the activity-dependent increase in *slowpoke* expression induced by several drugs directly correlates to an the increase in resistance to sedation that results in tolerance. This correlation holds true when *slowpoke* expression is induced by the same anesthetic, a different anesthetic or even when artificially induced using transgenic tools. It was observed that related drugs that display similar acute effects and induce expression of *slowpoke* will also show cross-tolerance. Cross-tolerance is the reduction in sensitivity to the effects of one drug after prior exposure to the another drug. In fact, cross-tolerance to benzyl alcohol was induced not only by chloroform, another solvent anesthetic; but was also induced by the non-solvent anesthetic CO_2. Both chloroform and CO_2, induce deep sedation in flies. On the contrary, the solvent toluene or a brief heat-shock, both of which cause hyperactivity in flies, will induce an increase in the duration of benzyl alcohol sedation after prior exposure to toluene or to a heat-shock (cross-sensitization).

These observations are clear indicators of homeostatic response to the effects of these treatments on neural excitability and not to drugs themselves and implicate

slowpoke as a key player in the homeostatic mechanism that results in drug tolerance.

Although, it is likely that *slowpoke* is one component of an orchestrated response of transcriptional changes and post-transcriptional modifications that involves many genes, sedation does not evoke this response from all ion channel genes. Neither the *Shaker* or *seizure* voltage-gated K^+ channel nor the *para* voltage-gated Na^+ channel transcripts shows a significant increase in expression following sedation. Although it is clear that mutations in several other ion channels genes affect the level of sensitivity to specific pharmacological effects of solvent intoxication, changes in the expression patterns of the other genes are not required for developing tolerance.

This data argues that the principle behind tolerance does not revolve around set levels of sensitivity or resistance towards a specific drug. Rather, tolerance implicates the capacity to produce significant changes in sensitivity in an experience dependent manner. Interestingly, the ion conductance for *slowpoke* channels are up to 10 times larger than all of the channels tested. A change in *slowpoke* alone would thus have a greater impact on the excitability of a neuron, making it a reasonable choice for generating large changes in cellular excitability at a lower transcriptional cost. Although the mechanisms by which the changes in activity translate into changes in *slowpoke* expression are still inconclusive, there is enough evidence to suggest that multiple signal transduction pathways are involved, including the well studied transcription factor CREB and the clock gene *period*.

The large-conductance calcium-activated potassium channels (BK), encoded by *slowpoke*, have been shown to participate in regulating neuronal firing patterns and

neurotransmitter release. Electrophysiological data presented here is consistent with the idea that increased BK channel expression from the *slowpoke* gene enhances net neural excitability and thereby helps the nervous system to resist sedation. Increased neural expression of *slowpoke* is, in some respect, a neural excitant, it augments the capacity for repetitive neural activity presumably by reducing the neural refractory period.

In conclusion, I postulate that the sedation-induced changes in *slowpoke* expression reflect a homeostatic mechanism that resists untoward changes in net cellular excitability. If hyperexcitability is induced, an activity-dependant mechanism alters channel expression to reduce this excitability, whereas if cellular excitability is suppressed, channel gene expression changes to enhance excitability. This homeostatic mechanism explains the development of tolerance to drugs that induce sedation.

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Vita

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