0145-6008/05/29010-1777$03.00/0
Alcoholism: Clinical and Experimental Research

Vol. 29, No. 10
October 2005

The *slowpoke* Gene Is Necessary for Rapid Ethanol Tolerance in *Drosophila*

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Background: Ethanol is one of the most commonly used drugs in the world. We are interested in the compensatory mechanisms used by the nervous system to counter the effects of ethanol intoxication. Recently, the *slowpoke* BK-type calcium-activated potassium channel gene has been shown to be involved in ethanol sensitivity in *Caenorhabditis elegans* and in rapid tolerance to the anesthetic benzyl alcohol in *Drosophila*.

Methods: We used *Drosophila* mutants to investigate the role of *slowpoke* in rapid tolerance to sedation with ethanol vapor. Rapid tolerance was defined as a reduction in the sedative phase caused by a single previous sedation. The ethanol and water contents of flies were measured to determine if pharmacodynamic changes could account for tolerance.

Results: A saturated ethanol air stream caused sedation in <20 min and resulted in rapid tolerance that was apparent 4 hr after sedation. Two independently isolated null mutations in the *slowpoke* gene eliminated the capacity for tolerance. In addition, a third mutation that blocked expression specifically in the nervous system also blocked rapid tolerance. Water measurements showed that both ethanol and mock sedation caused equivalent dehydration. Furthermore, a single prior exposure to ethanol did not cause a change in the ethanol clearance rate.

Conclusions: Rapid tolerance, measured as a reduction in the duration of sedation, is a pharmacokinetic response to ethanol that does not occur without *slowpoke* expression in the nervous system in *Drosophila*. The *slowpoke* channel must be involved in triggering or producing a homeostatic mechanism that opposes the sedative effects of ethanol.

Key Words: Ethanol, *Drosophila,* Rapid Tolerance, *slo*, *slowpoke*, BK Channels, Nervous System, Potassium Channel.

ALCOHOL IS THE one of the most commonly used drugs in the world second only to caffeine. It is also one of the most widely abused drugs, despite the fact that excessive drinking can lead to serious health risks, such as stroke, high blood pressure, cirrhosis of the liver, and cancer (Julien, 2004). People who are alcohol dependent also have an increased risk of mental disorders (Highlights From the Tenth Special Report to Congress, 2000), and >30% of traffic fatalities involve alcohol (Yi et al., 2004). The cost of alcohol abuse in the United States exceeds $200 billion a year (Harwood et al., 1992).

Like other addictive drugs such as cocaine, opioids, and amphetamines, alcohol acts on the mammalian mesolimbic dopamine system. This evolutionarily ancient system is part of the motivational system that regulates responses to natural reinforcers, including food, drink, sex, and social interactions. Addictive drugs cause an increase in firing of dopaminergic neurons in the ventral tegmental area of the midbrain, resulting in an increase in dopamine levels in the nucleus accumbens and other areas of the limbic system (Julien, 2004; Nestler, 2001a).

At low doses, the immediate effects of alcohol include hyperactivity, euphoria, and relief from anxiety and inhibitions. As the dose increases, there is a diminished response to sensory stimulation, reduced physical activity, loss of coordination and balance, and depression of cognitive functions. Even higher doses induce drowsiness, hypnosis, anesthesia, coma, and finally death (Julien, 2004).

Consumption of alcohol causes long-term physiological changes, including tolerance and, in some individuals, addiction. Tolerance is defined as a reduction of drug responsiveness caused by prior drug exposure. There are believed to be different types of tolerance. Acute tolerance occurs during the drug experience. Rapid tolerance is produced after the completion of a single drug experience, and chronic tolerance arises from serial drug exposures (Berger et al., 2004; Chao and Nestler, 2004). The acquisition of ethanol tolerance can lead to increased consumption, and

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Received for publication April 8, 2005; accepted July 22, 2005.

This material is based upon work supported by the National Science Foundation under Grant 0114716 (to N.S.A.) and a pilot grant from the Texas Commission on Alcohol and Drug Abuse (to N.S.A.).

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DOI: 10.1097/01.alc.0000183232.56788.62

this may speed addiction. Addiction is defined as a compulsive and uncontrolled use of a drug despite adverse consequences. Both tolerance and addiction are long-lasting phenomena that are believed to involve changes in gene expression (Chao and Nestler, 2004; McClung and Nestler, 2003; Nestler, 2001b).

Ethanol depresses signaling in the nervous system. The neuronal targets of ethanol feature a wide variety of ion channel proteins. In general, the acute effects of ethanol on the nervous system include the potentiation of inhibitory ion channels and the inhibition of excitatory ion channels (Harris, 1999). At physiologically relevant doses, ethanol has been shown to potentiate the activity of inhibitory ion channels including those gated by GABA, glycine, and serotonin and inwardly rectifying potassium channels. In addition, ethanol has been shown to inhibit the activity of stimulatory ion channels such as voltage-gated calcium channels, neuronal nicotinic acetylcholine receptor channels, and NMDA receptor channels (Crowder, 2004; Harris, 1999).

Another channel shown to be a target of ethanol is the BK-type calcium-activated potassium channel (Dopico et al., 1998; Jakab et al., 1997). This channel class is encoded by a single gene, called *slowpoke*, in both vertebrates and invertebrates. However, *slowpoke* transcripts undergo extensive alternative splicing, and the protein can be post-translationally modified, resulting in channels with different kinetics and calcium sensitivities (Adelman et al., 1992; Lagrutta et al., 1994; Tseng–Crank et al., 1994).

Ethanol has been shown to directly alter the activity of the *slowpoke* BK channel, and mutations in this channel have been linked to changes in alcohol and anesthetic sensitivity (Davies et al., 2003; Ghezzi et al., 2004; Leibovitch et al., 1995). Therefore, BK channels may be involved in both immediate and long-term effects of ethanol use. Mutations in the *C. elegans slowpoke* BK channel gene cause a decrease in ethanol sensitivity (Davies et al., 2003). Conversely, Leibovitch et al. (1995) demonstrated that *slowpoke* loss-of-function mutations enhance the sensitivity of flies to volatile anesthetics such as halothane. Tolerance can also be observed at the cellular level. In the mammalian hypothalamic/neurohypophysial system, it has been shown that synaptic BK channels rapidly become tolerant to the potentiating effects of ethanol (Pietrzykowski et al., 2004).

Recently, it was shown that in *Drosophila* sedation with the solvent anesthetic benzyl alcohol caused behavioral tolerance to the effect of the drug and concomitantly induced expression of the *slowpoke* gene; interestingly, null mutations in the *slowpoke* gene eliminated the capacity to acquire rapid tolerance, while induction of *slowpoke* gene expression in the absence of drug exposure was sufficient to cause a tolerant-like phenotype in a behavioral assay (Ghezzi et al., 2004). In humans, benzyl alcohol is used as an injectable local anesthetic and has been proposed to be a useful substitute for lidocaine (Wilson and Martin, 1999). Although benzyl alcohol has not been described as an abused drug, ethanol certainly is. In this study, we determined if ethanol tolerance in flies also shared the same relationship with the *slowpoke* gene. By testing a number of independently isolated mutant *slowpoke* alleles, we found that a functional *slowpoke* gene is required for the acquisition of rapid ethanol tolerance in *Drosophila*.

MATERIALS AND METHODS

Flies

Flies were raised on standard cornmeal/molasses/agar medium on a 12-hr/12-hr light dark cycle. Newly eclosed flies were collected over a 2-day period and studied when 5- to 7-days-old. Genotypes of stocks were as follows: Canton S; Oregon R; w^{1118}; slo^{4}; $ash2^{18}$/TM6B; Df(3R) crb87-5,st[1] e[1]/TM3; Sh^{KS133}; $para^{63}$/attached X; cac^{TS2}; and SK 3.2 and SK 7.2. Stocks carrying the SK 3.2 and SK 7.2 null mutations in the SK channel gene were a gift from J.P. Adelman and were generated by transposon mutagenesis (J.P. Adelman, personal communication). Only female flies were used except for the $para^{63}$ stock in which only males were used (*para* is on the X chromosome).

Inebriator

Ethanol was administered as a vapor in a glass and Teflon "inebriator." Air from a wall source was divided into two streams that passed through flowmeters at 15 ml/min. Each stream then traveled through a water bubbler (10-ml pipette through a stopper into 100 ml of distilled deionized water in a 250-ml Erlenmeyer flask). The fresh air stream lead directly to a treatment chamber. The other stream passed through two bubblers (Kontes Glass Co., Vineland, NJ, part number 737610-0000) containing 25 ml of 100% ethanol in a 65°C water bath, through a trap to collect any condensed ethanol, and then into a second treatment chamber. A valve could also be used to replace the ethanol vapor stream with a fresh air stream. Each treatment chamber consisted of six standard plastic vials of *Drosophila* that were clamped together. A manifold divided the incoming vapor stream into six individual vials. A fine mesh was placed over the end of each branch of the manifold to prevent flies from entering it. Eight holes were poked in the bottom of each vial with a heated 25-gauge needle to allow ethanol or air streams to exit the system. All tubing used was PTFE (Kontes Glass Co., Vineland, NJ). Both fresh air and ethanol air streams were delivered at the same velocity. All behavioral experiments were performed at the same time of day to reduce the effects of circadian rhythm upon behavioral activity.

Tolerance Assay

All behavioral experiments described here were performed with age- and sex-matched flies (5- to 7-day-old females) that were products of the same culture vials or bottles. The flies were divided into 12 vials of 10 flies each that were placed in one of two test chambers. One chamber received an air stream (control group) and the other received ethanol-saturated air (experimental group). The ethanol stream was replaced with a fresh air stream when all of the flies in the ethanol chamber were sedated. Sedated flies were scored as those that were lying on their backs or sides or those "facedown" with their legs splayed out in a nonstandard posture. When all flies in the experimental group had recovered, both groups were transferred to food vials. At a later time point, the flies were returned to the inebriator and sedated with ethanol. For the control animals, this was their first ethanol exposure, while for the experimental animals it was their second exposure. Vials for control and experimental groups were interdigitated in the chambers to minimize any position effect within the testing apparatus. Ethanol was administered until all flies were sedated and then replaced with fresh air. Tolerance was quantified during this second treatment by counting the number of recovered flies each minute. Some flies did

not recover during the observation period. However, they were accounted for statistically (see below). The results were graphed as the percentage of flies recovered from sedation. All experiments were done at the same time of day. The time course for ethanol tolerance was performed in the same manner except that the first treatment was performed in 2-liter plastic bottles that had been modified to accept the vapor and air inlets.

Statistics

The log-rank test for equality of survival was used to determine the significance between the recovery curves for animals sedated once or twice with ethanol. Survival analysis statistics were well suited to the analysis of time to a specific event, such as a recovery from sedation, and they accounted for animals that failed to respond during the analysis period (Hosmer et al., 2002). In all tolerance assays, the time that it took each fly to recover from sedation was measured. The statistic evaluated the entire recovery curves for the populations to determine significance (as opposed to individual data points that comprised the curve).

Gas Chromatography to Measure Ethanol Metabolism

To determine if prior ethanol treatment altered the rate of ethanol catabolism, age- and sex-matched w^{1118} and slo^4 flies were divided into two groups each. A group of w^{1118} and a group of slo^4 flies were sedated with ethanol in the inebriator. A second group of w^{1118} and slo^4 flies were exposed to fresh air in the inebriator. After the ethanol-exposed flies were sedated, they were switched to fresh air and allowed to recover. The control flies also remained in the treatment chambers during this recovery period. Once the sedated flies had recovered, all flies were transferred to food vials. Four hours later, all flies were returned to the inebriator, sedated with ethanol, and immediately removed from the chambers. This produced w^{1118} flies and slo^4 flies that had been treated once with ethanol and that had been treated twice with ethanol. Each of these four groups was subdivided into 15 food vials, five flies per vial. At $t = 0$, $t = 25$, $t = 50$, $t = 75$, and $t = 100$ min, three vials from each of the four groups were transferred to individual gas chromatography vials, sealed, and frozen. The weight of the vials before and after addition of the flies was noted. After all flies had been frozen, the vials were placed in a CP-3800 gas chromatograph (Varian, Inc., Palo Alto, CA) and ethanol levels were quantified against an ethanol standard curve. Molarity was determined using the measured water content of flies (see below).

Enzymatic Ethanol Assay

Sex- and age-matched wild-type flies were divided into groups of 10 flies each and sedated with ethanol in the inebriator. Twenty-four hours after ethanol sedation, each group of flies was homogenized in 1 ml of 50 mM Tris pH 7.5 and centrifuged to remove debris. Ethanol was quantified by mixing 5 μl of lysate with 1000 μl of Alcohol Reagent (Sigma-Aldrich, St. Louis, MO, part number 333–100), incubating for 10 min at room temperature, and measuring absorbance at 340 nm. Quantities were calculated using an ethanol standard curve. Molarity was determined using the measured water content of flies (see below).

Measurement of Water Content of Flies

The water content of 120 flies was estimated by weighing flies before and after desiccation at 65°C for 24 hr. We assumed that the reduction in mass caused by desiccation is almost completely due to water loss. To determine whether the inebriator caused dehydration, flies were weighed before and after passage through the inebriator. For all water measurements $n = 6$.

RESULTS

Sedation of Flies Using Ethanol Vapor

We previously reported that flies sedated with volatile solvent inhalants/anesthetics acquire rapid tolerance after a single sedation and that the *slowpoke* gene is involved in the manifestation of this phenotype (Ghezzi et al., 2004). Therefore, we determined if a similar relationship existed between *slowpoke* and the acquisition of rapid tolerance to ethanol. We used the previous experimental paradigm as a basis, making changes to account for differences in the physical properties of and the behavioral responses to ethanol and benzyl alcohol.

In the earlier study, benzyl alcohol was administered by allowing the flies to come into contact with a fine coating of the solvent on the interior of a vial. However, ethanol has a higher vapor pressure and thus cannot be used in this manner (Ghezzi et al. 2004). Others who study the effects of ethanol in *Drosophila* have used ethanol-saturated air streams as a means to deliver a reproducible ethanol dose and to rapidly sedate flies (Moore et al., 1998). A major advantage of vapor administration over administration by injection or feeding is that a sedating dose can be rapidly and equally delivered to a large population. We adopted this standard methodology.

During the first few minutes of ethanol exposure, the flies enter a hyperexcitable phase, in which they walk more and at a greater speed. This hyperkinetic phase persists for a few minutes, after which movement subsides and eventually stops. After ≈10 minutes of ethanol exposure, flies become sedated. This sequence of responses has been described in remarkable detail by Rothenfluh and Heberlein (2002) and is similar to the response to other volatile solvents (Ghezzi et al., 2004). We scored sedated flies as those that were lying on their backs or sides or those "facedown" with their legs splayed out in a nonstandard posture.

We observed that flies responded differently to ethanol than to the other solvents with which we were familiar (Ghezzi et al., 2004). A minor difference between the sedation by ethanol and the sedation with other solvents such as benzyl alcohol (Ghezzi et al., 2004) is that flies sedated with ethanol did not become completely immobile. For most ethanol-sedated flies, we observed occasional twitching of the legs. Others also noted that even heavily sedated flies continue to twitch their legs (Dzitoyeva et al., 2003). Casual observation indicated that this was suppressed only by ethanol doses that resulted in some lethality.

A more significant difference in the behavior of ethanol sedated flies and flies sedated with other solvents exists during the recovery phase. During a typical treatment, a group of flies is exposed to a stream of ethanol-saturated air just until the last fly has been sedated. At that point, the ethanol stream is replaced with a stream of fresh air to clear the vial of any residual ethanol vapor. After their return to

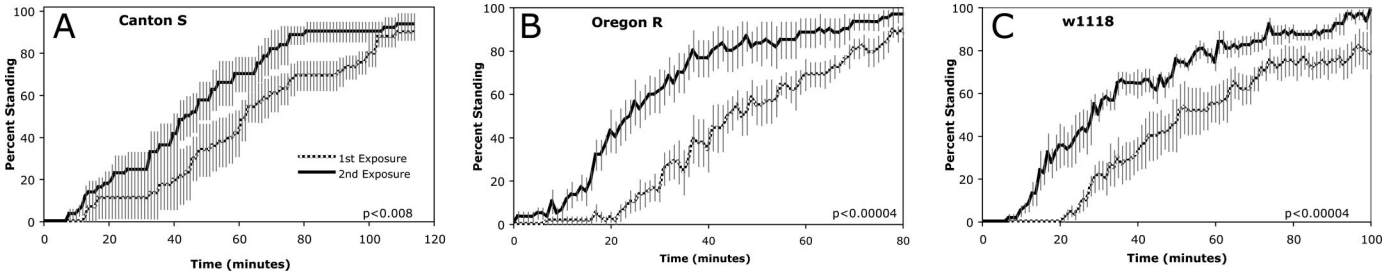

Fig. 1. Tolerance in three common wild-type laboratory strains of *Drosophila*: Canton S, Oregon R, and w^{1118}. Recovery curves for a population of age-matched females after their first (gray curve) and second (black curve) ethanol sedation. Four hours elapsed between the first and second exposures. Values are the percentage of flies with postural control (standing or walking). Counts were made at 1-min intervals. A significant difference between the curves was determined using the log-rank test ($n = 60$); however, the error bars are SEM for each data point. A representative experiment is shown.

fresh air, the animals require ≈1 to 2 hr for the entire population to recover. *Drosophila* flies are negatively geotactic, meaning that they climb up the walls of their vials. Ghezzi et al. (2004) used the resumption of wall climbing as an indicator of recovery from benzyl alcohol sedation. However, flies sedated with our ethanol apparatus did not immediately resume wall climbing after recovery. Instead, the flies stood and fed, but they walked little. Therefore, in the current study, flies were said to have recovered from ethanol sedation when they stood upright and appeared to have regained postural control. We recognize that standing does not indicate that the animals have fully recovered from sedation. However, this behavior is merely an easily measurable landmark of the recovery process.

Quantification of Tolerance

A variety of techniques have been used to show that *Drosophila* flies acquire rapid tolerance to the effects of ethanol (Berger et al., 2004; Dzitoyeva et al., 2003; Scholz et al., 2000) We quantified ethanol tolerance as a reduction in the period of sedation caused by prior ethanol exposure. Control and experimental flies were transferred to the inebriator, and the experimental flies were sedated with ethanol. Four hours after the first sedation, the flies were returned to the inebriator, and all were sedated with ethanol. For the control animals, this was their first ethanol exposure, while for the experimental animals it was their second exposure. Ethanol was administered until all flies were sedated (<20 min) and then replaced with fresh air. During this recovery phase, we counted the number of flies that were standing with postural control once every minute from the time that the ethanol was first applied. We measured behavioral tolerance at both 4 and 24 hr after the first ethanol exposure.

Figure 1 compares the recovery curves for three different laboratory stocks sedated one time and two times with ethanol. The curves show the percentage of animals that were not sedated (standing) every minute. For each curve, ≈60 animals were used (the occasional animal was lost or died before it could be retested).

In the study, we only analyzed the recovery curves. The knockdown or sedation phase takes place over a shorter period (minutes vs. hours). It was technically less demanding to use the recovery curves (a measure of the length of time that a population was sedated) to detect tolerance than to use the corresponding knockdown curves.

The absolute shape and time course of the recovery curves varied on a daily basis, with changes in the weather and between stocks. This is not unexpected in a behavioral assay of this nature. To account for this, direct comparisons between recovery curves were made only for flies that were age and sex matched, isolated from the same vials, and assayed in tandem at the same time.

The recovery curve for animals experiencing their second ethanol sedation was leftward shifted with respect to the recovery curve for animals experiencing their first ethanol sedation. Tolerance was defined as a statistically significant leftward shift in the recovery curve caused by prior ethanol exposure. Statistical significance was determined using log-rank survival analysis (Ghezzi et al., 2004; Hosmer et al., 2002). This test is well suited to time-to-event measurements and takes into account the shape of the entire curve. We did not attempt to compare the degree of tolerance between stocks because genetic background seemed to affect its absolute magnitude. At this time, we preferred to treat it as a binary event—that is, either a population showed tolerance (a significantly different leftward-shifted recovery curve) or it did not.

We also studied only responses that were not overly dependent on genetic background but that could be easily seen in a variety of stocks. Thus, in our behavioral assays, we tested common laboratory stocks and did not use isogenic stocks. Figure 1 shows the recovery profiles of three different standard laboratory stocks: Canton S, Oregon R, and w^{1118}. All three showed robust and highly significant tolerance.

Time Course of Rapid Tolerance

To determine how long tolerance persisted, a large number of matched Canton S flies were divided into two groups. The flies were placed in 2-liter treatment chambers; one group was exposed to ethanol vapor until sedated, while the other group was exposed to a stream of fresh air. The flies

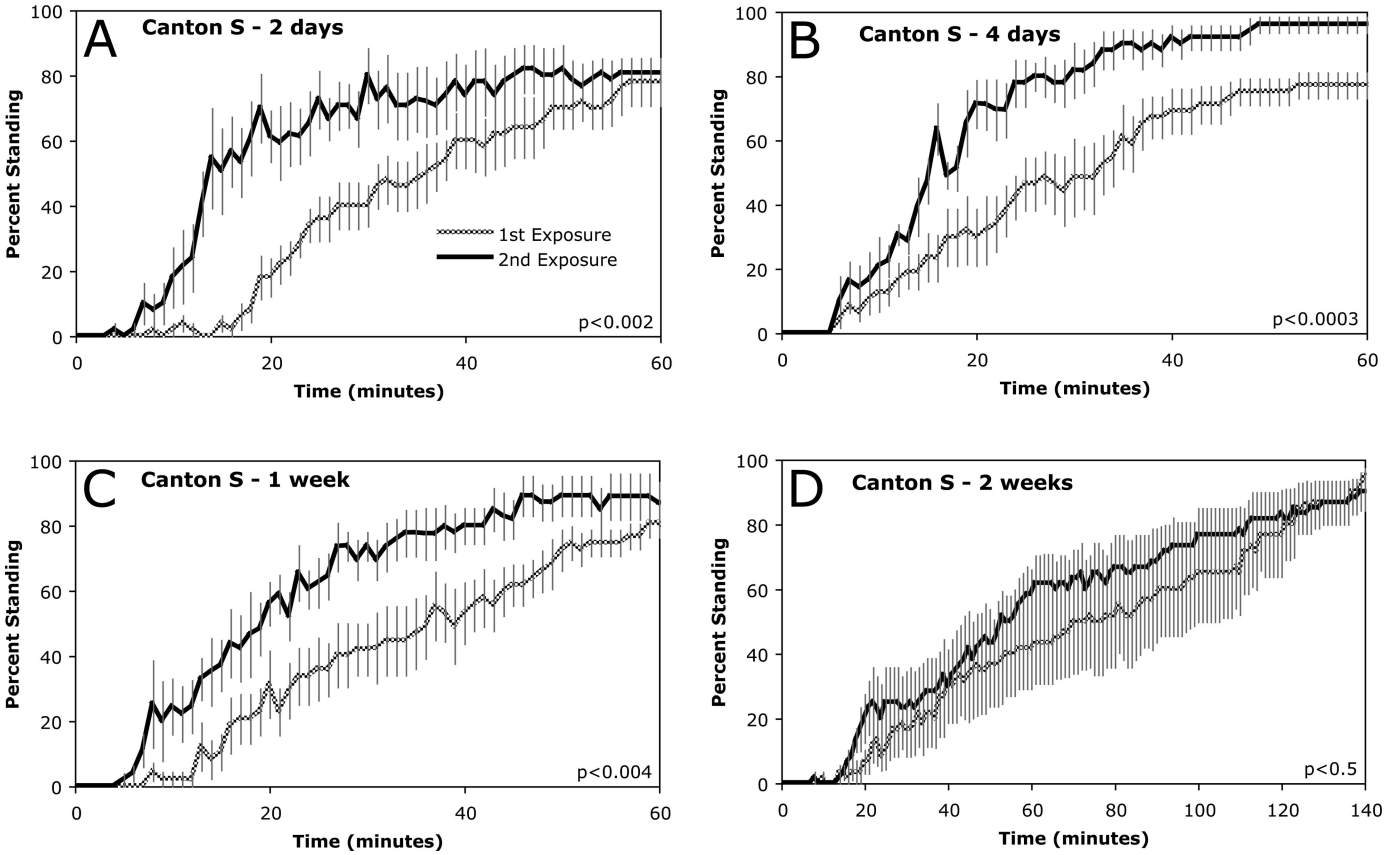

Fig. 2. Time course of tolerance. A large population of age-matched female flies was split into two groups. One group was sedated with an ethanol-saturated air stream, while the other group was simultaneously exposed to a stream of fresh air. Two days (A), 4 days (B), 1 week (C), and 2 weeks (D) later, 50 flies from each group were sedated with an ethanol-saturated air stream. The ethanol air stream was replaced with fresh air as soon as all flies were sedated, and the recovery of the populations was monitored at 1-min intervals. Significance between the curves was determined using the log-rank test ($n = 50$), while the error bars are SEM for each data point. Tolerance was obvious after 2 days (A), 4 days (B), and 1 week (C). Tolerance was no longer apparent at the 2-week time point (D). The entire time course experiment was performed a single time.

were then divided into smaller groups and stored for 2, 4, 7, or 14 days in food vials. The animals were then tested for ethanol tolerance using the inebriator as described above. Tolerance persisted for at least 7 days, but it was not apparent 14 days after the first ethanol sedation (Fig. 2).

Mutations in the slowpoke Gene Eliminate Rapid Tolerance

The slo^4 mutation is a chromosomal inversion with a break point within the gene. It is thought to be a null mutation based on the following: genetic complementation tests, failure of RT-PCR and northern analyses to detect mRNA, failure of immunohistochemical staining to detect a protein, and failure of electrophysiological assays to detect BK channels in muscle (Atkinson et al., 1991; Becker et al., 1995). We observed that flies homozygous for the slo^4 mutation were unable to acquire ethanol tolerance in response to a single ethanol sedation (Fig. 3A), suggesting that a functional *slowpoke* gene is required for the acquisition of rapid tolerance.

However, it is possible that it is not the loss of the *slowpoke* channel that blocks tolerance. The slo^4 mutation might produce a truncated polypeptide that has eluded detection, and this product might interfere with the capacity of the flies to acquire tolerance in a dominant negative manner.

To test this contingency we crossed homozygous slo^4/slo^4 flies to the wild-type strain Canton S to generate heterozygous $slo^4/+$ flies. These flies retained the ability to acquire tolerance (Fig. 3B), indicating that the loss of tolerance is not due to a dominant phenotype associated with the slo^4 chromosome. This also shows that a single functional copy of the *slowpoke* gene is sufficient for the acquisition of tolerance.

An alternative hypothesis is that the slo^4 line of flies carries a second unidentified mutation or an allelic variant(s) that causes the animals to be unable to acquire tolerance. To evaluate this possibility, we tested other mutant lines that carry different, independently isolated genetic lesions that interfere with *slowpoke* expression. If the loss of *slowpoke* activity is the cause of the phenotype, then these mutations should also block the acquisition of tolerance. The deficiency strains Df(3R)crb 87-5/TM3 and $ash2^{18}$/TM3 both carry large deletions on the third chromosome that involve the *slowpoke* gene. The Df(3R)crb 87-5 deletion removes the *slowpoke* gene and thus eliminates expression in all tissues (Atkinson et al., 1991), whereas with respect to *slowpoke* the $ash2^{18}$ deletion is a

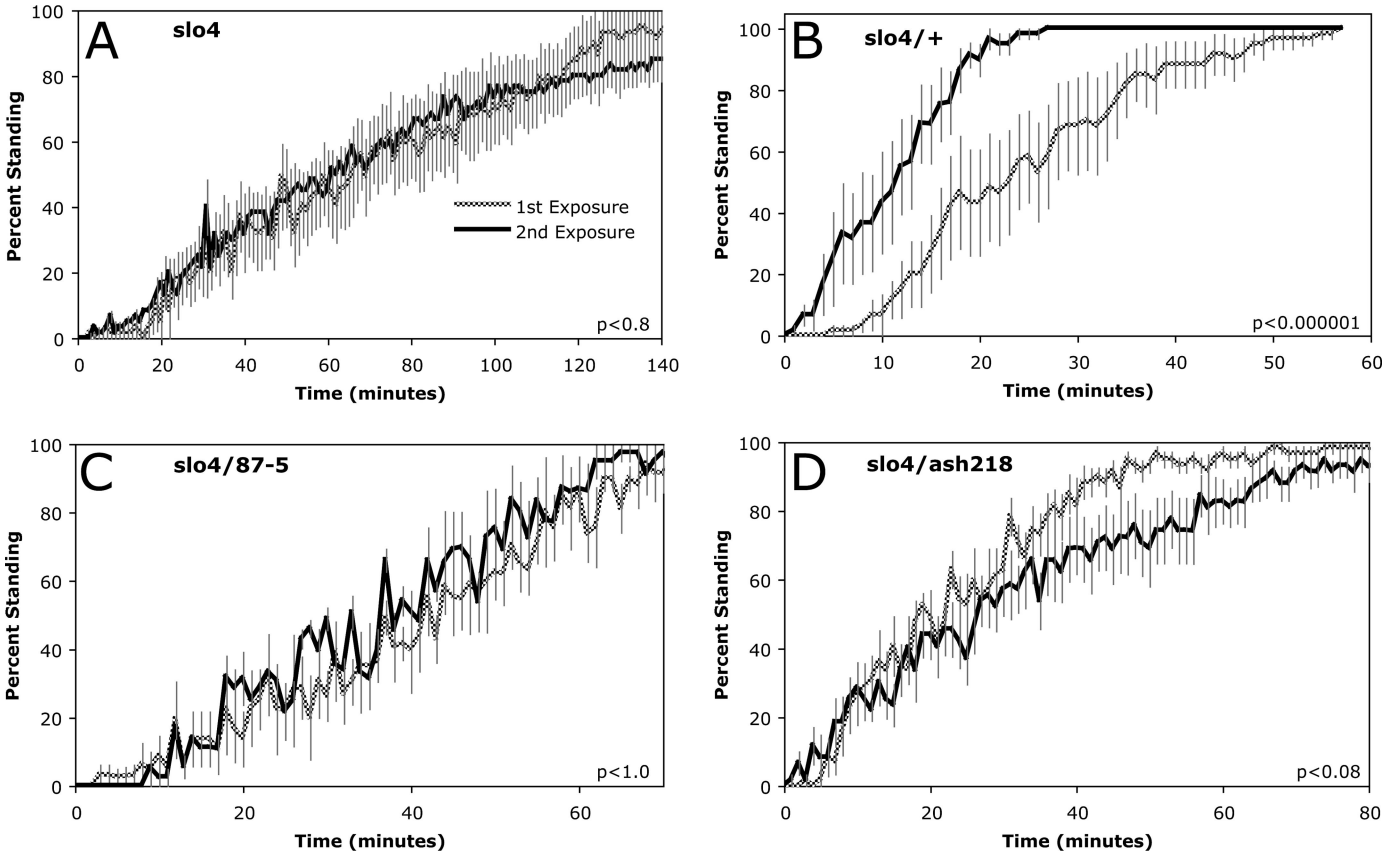

Fig. 3. *slowpoke* mutants did not acquire tolerance. Recovery curve after one (gray line) and two (black line) rounds of ethanol sedation. (A) The slo^4 mutation, a homozygous viable null mutation in the *slowpoke* gene, prevented the appearance of tolerance. (B) Animals heterozygous for slo^4 acquired tolerance, indicating that the chromosome does not contain a dominant mutation that interferes with tolerance. (C) Recovery curves for S87-5/slo^4 transheterozygotes, indicating that the S87-5 deletion of the *slowpoke* gene interferes with the capacity of the animal to acquire tolerance. (D) The transheterozygous combination of the $ash2^{18}$ deletion and the slo^4 allele eliminated *slowpoke* expression in the nervous system and prevented the acquisition of tolerance. Significance between the curves was determined used the log-rank test ($n = 60$), while the error bars are SEM for each data point. A representative experiment is shown.

promoter mutant. The *slowpoke* gene has five tissue-specific promoters, two of which are responsible for neural expression. The $ash2^{18}$ deletion removes the two neural promoters and eliminates neural expression of *slowpoke*, while leaving muscle expression intact (Atkinson et al., 2000).

Each of these deletion chromosomes is homozygous lethal and thus cannot be tested directly. Lethality is thought to arise because of the loss of genes other than *slowpoke* (The Flybase Consortium, 2003). To determine whether these lesions also prevent the acquisition of tolerance, we crossed each of the deficiency stocks to the homozygous slo^4 stock to produce animals with the genotype Df(3R)crb 87-5/slo^4 and the genotype $ash2^{18}/slo^4$. Neither animals with the genotype Df(3R)crb 87-5/slo^4 nor animals with the genotype $ash2^{18}/slo^4$ were able to acquire tolerance (Fig. 3, C and D), indicating that the ability to acquire ethanol tolerance is dependent on a functional *slowpoke* gene. Furthermore, because the $ash2^{18}/slo^4$ animals have lost only expression of *slowpoke* in the nervous system, we can conclude that it is in the nervous system that *slowpoke* plays a role in mediating rapid tolerance.

Prior Ethanol Sedation Does Not Alter the Rate of Ethanol Clearance

The tolerance observed in the wild-type flies could reflect the adaptation of the nervous system to the presence of the drug (functional tolerance) and/or an enhanced capacity to catabolize ethanol (metabolic tolerance). In flies, like humans, alcohol dehydrogenase is responsible for most ethanol metabolism. It has been reported that ethanol consumption does not cause an increase in alcohol dehydrogenase expression in the adult (Geer et al., 1988).

However, other changes in metabolism, including a change in the respiratory rate, could pharmacokinetically contribute to rapid tolerance. To account for all possibilities, we measured the rate at which wild-type and slo^4 stocks metabolized ethanol. We also tested if prior exposure to ethanol changed the rate of ethanol clearance. To do so, we used gas chromatography to measure the absolute amount of ethanol in the flies at different time points throughout the recovery phase. The weight of each fly was also noted before decomposition for gas chromatography.

We made the assumption that the ethanol in a fly was dissolved in aqueous solution. To express ethanol content

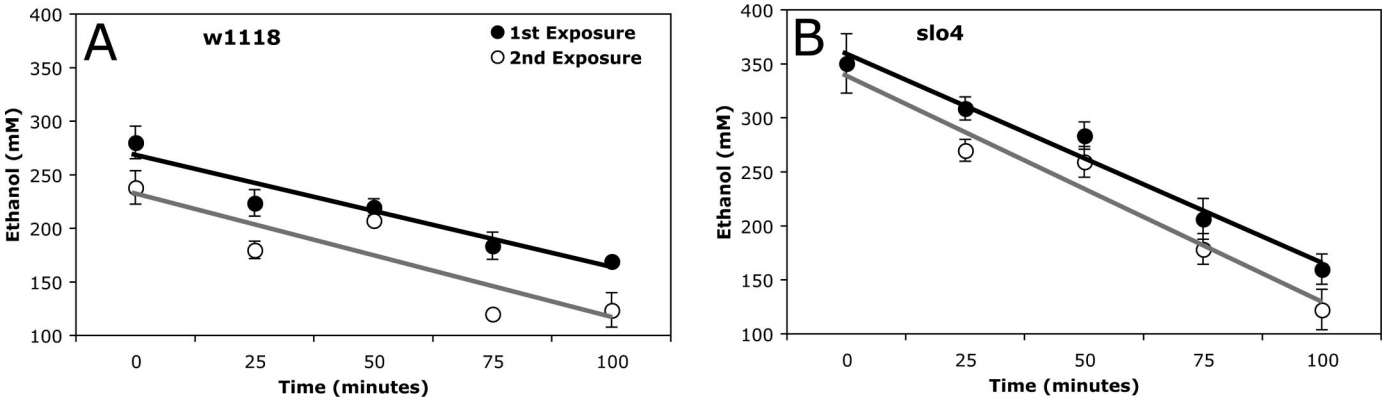

Fig. 4. Prior ethanol exposure did not alter the rate of ethanol metabolism. The catabolism of ethanol was measured in w^{1118} and slo^4 homozygotes after their first ethanol exposure and after their second exposure. (A) w^{1118} flies were assayed 0, 25, 50, 75, and 100 min after their first exposure (filled circles, black line) and second exposure (open circles, gray line). (B) The filled circles and the black line represent the metabolism of ethanol in slo^4 flies at 0, 25, 50, 75, and 100 min after their first sedation. The open circles and the gray line represent the metabolism of ethanol in slo^4 flies after their second sedation at 25, 50, 75, and 100 min after sedation. The lines represent the line of best fit. Ethanol content of the flies was determined by gas chromatography using an ethanol standard curve ($n = 6$). Error bars represent SE of the mean. Water content of the flies was measured to be 0.98 ± 0.06 μl before treatment and 0.88 ± 0.03 μl after treatment (see text).

of the flies as a concentration, we measured the water in the flies. Other ethanol researchers have estimated that male flies contain 2 μl of water (Scholz et al., 2000). However, our measure of water content showed that before ethanol sedation the larger female contained an average of 0.98 ± 0.06 μl of water (65% of body weight). This measurement is in close agreement with measurements made by others (Folk and Bradley, 2004; Telonis–Scott and Hoffmann, 2003). We also observed that the inebriator caused substantial dehydration. The average water content 1 hr after treatment was 0.88 ± 0.03 μl. The 15-20 min spent in the inebriator caused the flies to lose on average 0.10 μl of water. Twenty four hours later, control flies regained the lost water and had an average water content of 0.97 ± 0.02 μl, while ethanol-sedated flies still had an average water content of 0.88 ± 0.03 μl (for all water measurements, $n = 6$).

Interestingly, this water loss occurred whether the flies were exposed to the ethanol-saturated air stream or to the fresh air stream. Using these values, gas chromatography showed that immediately after their second sedation that wild-type females were ≈240-280 mM ethanol. To confirm this value, we repeated this measure using an enzymatic assay for ethanol. The second assay gave an average value of 235 mM. Figure 4 shows that the decay rate of ethanol was not altered by prior ethanol sedation in either wild-type or slo^4 stocks. Thus, the capacity to acquire tolerance cannot be accounted for by a pharmacokinetic mechanism.

Rapid Tolerance Is Not Prevented by Mutations in Other Channel Genes

It is possible that tolerance is a delicate phenotype and that any mutation that perturbs neural signaling will prevent the acquisition or maintenance of tolerance. To test this idea, we examined whether mutations in other potassium channel genes interfere with the acquisition of tolerance. Animals homozygous for null mutations in the *Shaker* potassium channel gene (Sh^{KS133}) or the SK calcium-activated potassium channels (SK 3.2 and SK 7.2) were tested for the capacity to acquire tolerance. In response to a single ethanol sedation, all of these mutant lines exhibited a robust rapid tolerance response (Fig. 5, A and B).

In addition, we tested animals carrying mutant alleles of the *paralytic* ($para^{63}$) voltage-activated sodium channel gene and the cacophony (cac^{ts2}) L-type voltage-activated calcium channel gene. Neither of these two are null mutations; however, both mutations have substantial effects on neural signaling. The $para^{63}$ allele is a hypomorphic mutant allele (Stern et al., 1990), while animals homozygous for cac^{ts2} mutation show some behavioral defects even at the permissive temperature (Kawasaki et al., 2000; The Flybase Consortium, 2003). These mutant lines also mounted a strong tolerance phenotype in response to a single ethanol sedation. This indicates that the *slowpoke* mutation is somewhat special in its ability to interfere with the acquisition of tolerance.

DISCUSSION

Great strides have been made in developing *Drosophila* as a model system for the study of drugs of abuse (Dzitoyeva et al., 2003; Wen et al., 2005; Wolf and Heberlein, 2003). It has been shown that many abused drugs act through the same neurotransmitter systems in both *Drosophila* and mammals (Bainton et al., 2000; Porzgen et al., 2001). These similarities between *Drosophila* and mammals give credence to the use of the *Drosophila* model system as a gene discovery engine to identify genes involved in immediate and long-term responses to alcohol. Classical genetic screens have produced a number of novel genes whose characterization will open new avenues for understanding the effects of alcohol (Berger et al., 2004). The *Drosophila* system also has a large number of preexisting single gene mutations that alter the behavior and responses of the animals to their environment. These provide excel-

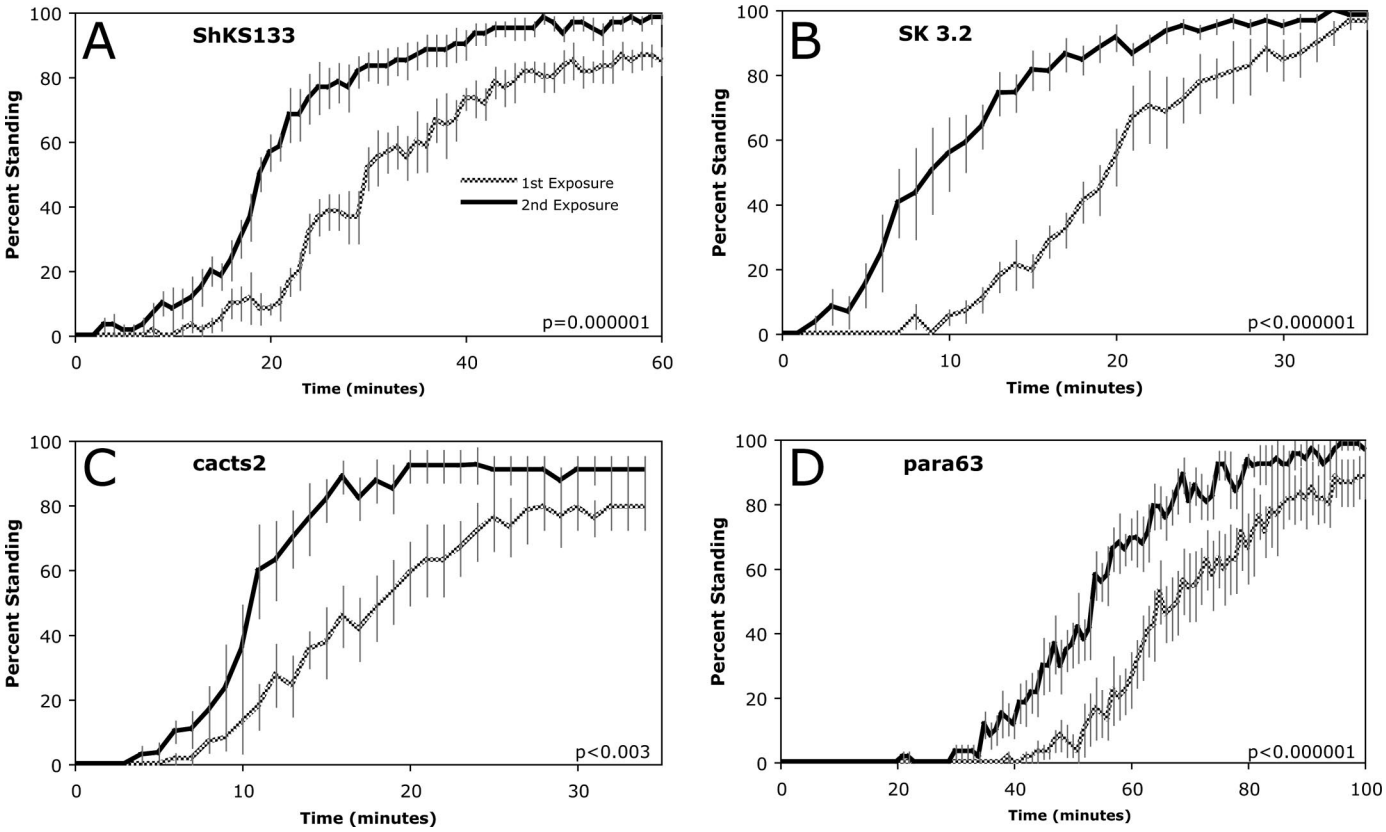

Fig. 5. Flies homozygous for mutant alleles of the *Shaker* voltage-gated potassium channel, the SK calcium-activated potassium channel, the L-type voltage-gated calcium channel, or the voltage-gated sodium channel retained the ability to acquire ethanol tolerance. Ethanol sedation recovery curves were made as described in Fig. 1 using female flies, except for the curve in (D), which because the *paralytic* gene is on the X chromosome required the use of male flies. (A) The Sh^{KS133} is a mutation in the *Shaker* (Kv1.1) voltage-gated potassium channel. (B) SK 3.2 is a transposon-mediated mutation in the small-conductance calcium-activated potassium channel gene. A second mutant allele, SK 7.2, was also shown to acquire tolerance (data not shown). (C) cac^{ts2} is the mutant allele of the cacophony voltage-gated calcium channel gene. (D) $para^{63}$ is a hypomorphic allele of the *paralytic* voltage-gated sodium channel gene. Statistical significant differences between the recovery curves of flies experiencing their first and second ethanol treatment were determined using the log-rank test of significance ($n = 60$ for $para^{63}$, *Sh*, and SK 3.2; $n = 40$ for cac^{ts2}), while the error bars are SEM for each data point. A representative experiment is shown.

lent tools for rapidly testing genes for a role in the response of the animal to alcohol.

The *slowpoke* gene encodes the BK-type large-conductance potassium channel that is activated by both intracellular calcium and membrane depolarization. BK channels are widely distributed throughout both excitable and nonexcitable cells (Calderone, 2002). In the mammalian brain, they are abundant in the hippocampus, cerebellum, thalamus, amygdala, neocortex, and olfactory cortex (Gribkoff et al., 2001). BK channels have been shown to regulate action potential shape, duration, and frequency as well as neuronal excitability and transmitter release (Calderone, 2002; Gho and Ganetzky, 1992; Gribkoff et al., 2001; Lancaster and Nicoll, 1987; Robitaille et al., 1999; Sah and McLachlan, 1992). In addition, BK channels are also abundant in smooth muscle and endocrine tissue, where they control muscle tone and contractility, and neuroendocrine secretion (Vergara et al., 1998).

A null mutation in the *slowpoke* gene causes a number of small changes in *Drosophila* behavior; *slowpoke* flies exhibit a stimulus-induced sticky-feet phenotype, a chronic reduction in the capacity for flight, a change in the pattern of the male courtship song, and a suppression of circadian rhythms (Atkinson et al., 2000; Ceriani et al., 2002; Elkins et al., 1986; Peixoto and Hall, 1998). In mammals, mutations in this gene are also associated with modest changes in behavior (Meredith et al., 2004). This is surprising because it is believed that all BK-type calcium-activated potassium channels are encoded by a single *slowpoke* gene, and hence one would anticipate that the complete loss of all large-conductance calcium-activated potassium channels would have more profound consequences.

The *slowpoke* gene has been previously implicated in drug responsiveness in the Caenorhabditis and *Drosophila* invertebrate model systems (Davies et al., 2003; Ghezzi et al., 2004). In *C. elegans*, ethanol appears to directly modulate channel activity, and the ethanol sensitivity of the animal is inversely correlated to the level of expression from the gene (Davies et al., 2003). In *Drosophila*, it has been observed that a single sedation with the solvent anesthetic benzyl alcohol induces *slowpoke* gene expression and also produces rapid tolerance to this anesthetic. Mutations in the *slowpoke* gene blocked the production of tolerance, while transgenic induction of *slowpoke* appears to be sufficient to reproduce the tolerance phenotype (Ghezzi et al., 2004).

Benzyl alcohol is a compound that has primarily been used as a contact anesthetic. Medically, it has been used as a substitute for lidocaine. It is not at all clear that responses to benzyl alcohol and ethanol will be molecularly related. Furthermore, the behavioral responses to the solvent anesthetic benzyl alcohol and to ethanol differ slightly, suggesting that ethanol and benzyl alcohol tolerance might not depend on the same genes. Here, we showed that in *Drosophila* a null mutation in the *slowpoke* gene completely eliminated the acquisition of rapid ethanol tolerance.

We used an ethanol-saturated air stream to sedate flies. This resulted in rapid sedation by a very high dose of ethanol. By measuring the alcohol in the flies, we estimated its hemolymph concentration. In the fly, our treatment yielded a physiological concentration that was at least twice the lethal blood alcohol concentration in a human (Julien, 2004). Our values were substantially higher than those reported by others (Scholz et al., 2000). This may arise from differences in the estimated versus measured water content of flies, the treatment paradigm, and the husbandry conditions. In any case, it is clear that *Drosophila* flies survive much higher concentrations of ethanol than mammals. Nevertheless, we do not believe that this compromises their use as a model system for the study of responses to alcohol. A difference in ethanol sensitivity between mammals and insects should be not surprising because *Drosophila melanogaster* flies use fermenting substrates as sites for breeding and food and therefore are well adapted to high concentrations of ethanol (Ashburner, 1998). Furthermore, it has been observed that *Drosophila* flies are much more resistant to a wide variety of insults than mammals, including radiation and anoxia (Ma and Haddad, 1997; Wharton and Wharton, 1959). Despite these differences, most of the basic regulatory mechanisms governing the behavior of eukaryotic cells are conserved between *Drosophila* and mammals.

Null mutations in *slowpoke* block the acquisition or maintenance of functional ethanol tolerance. This is clearly a neural phenotype because a promoter mutant ($ash2^{18}$) that eliminates *slowpoke* expression only in the nervous system also fails to display tolerance. Furthermore, the fact that other channel mutants did not compromise the capacity for ethanol tolerance suggests that the *slowpoke* gene plays a special role in this process and that the inability to acquire tolerance is not merely the product of a generic disturbance in neural signaling. Future manipulation of these and other *slowpoke* mutants may lead to the identification, in the central nervous system, of key neural signaling components required for the production of rapid tolerance.

Mutations in *slowpoke* might interfere with the tolerance machinery in a number of ways. The loss of rapid ethanol tolerance phenotype could reflect an inability to trigger or to maintain tolerance. It may be that a large dose of ethanol modifies the activity of the *slowpoke* channel and that this change is the trigger for the induction of tolerance. In some systems, the activity of *slowpoke* channels has been shown to be directly modulated by ethanol; however, this has not yet been demonstrated in *Drosophila*. This possibility is in concordance with the observation that *slowpoke* mutants in *C. elegans* limit the effects of ethanol, making the animals more resistance to intoxication (Davies et al., 2003).

However, the inability to trigger or maintain tolerance might also arise from changes in the development of the nervous system caused by the complete absence of the *slowpoke* channel. Finally, *slowpoke* channels may not be involved in triggering tolerance but be a component of a homeostatic mechanism, limiting changes in neural activity caused by environmental factors. Certainly, pharmacokinetic drug tolerance is likely to be a complex response of the nervous system, requiring the coordinated activity of a large number of cells and a large number of genes. We do not believe that *slowpoke* is the only gene involved in this process. Others have identified additional genes that also perturb the capacity for tolerance in flies (Berger et al., 2004; Dzitoyeva et al., 2003; Park et al., 2000; Wen et al., 2005), and these may all be part of the same biochemical process that underlies behavioral tolerance. Finally, there exist other mechanistically distinct forms of alcohol tolerance (e.g., chronic tolerance) that can be induced by different drug regimens. It will be interesting to see if *slowpoke* plays a central role in all forms of ethanol tolerance or if it is specific to rapid tolerance (Berger et al., 2004).

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