Ethanol Tolerance Caused by *slowpoke* Induction in *Drosophila*

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Background: The large-conductance calcium-activated potassium channel encoded by the *slowpoke* gene has recently been implicated in the ethanol response. *Caenorhabditis elegans* carrying mutations in this gene have altered ethanol sensitivity and *Drosophila* mutant for this gene are unable to acquire rapid tolerance to ethanol or anesthetics. In *Drosophila*, induction of *slowpoke* expression has been linked to anesthetic resistance.

Methods: We used *Drosophila* as a model system to examine the relationship between *slowpoke* expression and ethanol tolerance. Real-time PCR and a reporter transgene were used to measure *slowpoke* induction after ethanol sedation. An inducible *slowpoke* transgene was used to manipulate *slowpoke* levels in the absence of ethanol sedation.

Results: Ethanol sedation increased transcription from the *slowpoke* neural promoters but not from the *slowpoke* muscle/tracheal cell promoters. This neural-specific change was concomitant with the appearance of ethanol tolerance, leading us to suspect linkage between the two. Moreover, induction of *slowpoke* expression from a transgene produced a phenotype that mimics ethanol tolerance.

Conclusions: In *Drosophila*, ethanol sedation induces *slowpoke* expression in the nervous system and results in ethanol tolerance. The induction of *slowpoke* expression alone is sufficient to produce a phenotype that is indistinguishable from true ethanol tolerance. Therefore, the regulation of the *slowpoke* BK-type channel gene must play an integral role in the *Drosophila* ethanol response.

Key Words: *Drosophila*, *slowpoke*, BK Channel, Tolerance, Ethanol.

ALCOHOL ABUSE CAN cause a myriad of deleterious effects including neuropathy, cardiomyopathy, gastritis, pancreatitis, altered hormone release, hypothalamic neuronal degeneration, and cirrhosis of the liver (Beers and Berkow, 1999; Madeira and Paula-Barbosa, 1999). Despite knowledge of the link between these effects and excess use of alcohol, people are driven to abuse alcohol because of 2 poorly understood phenomena, tolerance and addiction. Tolerance is defined as a reduced response to a drug with repeated exposure, whereas addiction is a compulsive pattern of drug use regardless of adverse consequences. These phenomena are thought to involve long-term changes in many neuronal proteins in different regions of the brain.

It has been demonstrated that alcohol directly interacts with certain ion channels in the brain, altering their activity (Harris, 1999). In general, alcohol causes the inhibition of excitatory ion channels, such as *N*-methyl-D-aspartate (NMDA) receptors and L-type voltage-gated calcium channels, and the potentiation of inhibitory ion channels, such as γ-aminobutyric acid $(GABA)_A$ and glycine receptors. This leads to an overall depression of the nervous system and is likely responsible for the symptoms associated with alcohol intoxication such as euphoria, the relief from inhibitions, loss of coordination, and depression of cognitive functions (Julien, 2004).

Many of the same ion channels whose activity is directly modulated by ethanol have also been shown to have their expression regulated by chronic ethanol exposure, generally in a manner opposite to the acute effect. For instance, chronic ethanol exposure causes an up-regulation of NMDA receptors in hippocampal explants (Thomas and Riley, 1998) and also up-regulates NMDA R1 and R2B polypeptide subunits in cortical neurons (Follesa and Ticku, 1996). Chronic ethanol exposure also leads to an increase in the number of L-type voltage-gated calcium channels in rat inferior colliculus neurons (N'Gouemo and Morad, 2003) and in the neurohypophysis (Knott et al., 2002). Conversely, chronic treatment causes a down-regulation of the $GABA_A$ α1 subunit in the amygdala and cerebral cortex (Kumar et al., 2003; Papadeas et al., 2001)

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and the α4 subunit in the amygdala and nucleus accumbens (Papadeas et al., 2001).

These changes in expression of ion channel genes may be a compensatory mechanism geared to restore appropriate excitability to the nervous system. Unfortunately, upon cessation of alcohol exposure, these homeostatic modifications can themselves lead to aberrant neural activity. For example, animals accustomed to high levels of ethanol consumption often have seizures if the administration of ethanol is abruptly terminated (Saitz, 1998). These seizures have been shown to be reduced by inhibiting NMDA receptors (Morrisett et al., 1990) and L-type calcium channels (Walter and Messing, 1999). Ethanol-induced changes in channel expression, and the resulting changes in neural activity, may contribute to the phenomena of tolerance and addiction.

An ion channel of recent interest in the field of alcohol addiction is the large-conductance calcium-activated potassium channel encoded by the *slowpoke* gene, also known as the BK or Maxi-K channel. It is expressed widely throughout excitable tissues, where it affects action potential shape, neural excitability, and transmitter release, as well as smooth muscle tone and neuroendocrine secretion (Gribkoff et al., 2001). The gene encoding this channel was recently identified in a *Caenorhabditis elegans* mutant screen for altered ethanol sensitivity (Davies et al., 2003), and the activity of the channel has been shown to be directly modulated by ethanol. It is potentiated by acute doses of alcohol in clonal pituitary cells (Jakab et al., 1997), rat neurohypophysial terminals, and dorsal root ganglion neurons (Liu et al., 2003). Further, this appears to be an intrinsic property of the channel since it is also potentiated by alcohol in isolated membrane patches from skeletal muscle, when heterologously expressed in *Xenopus* oocytes, and when incorporated into planar lipid bilayer membranes (Chu et al., 1998; Dopico et al., 1998). However, ethanol failed to potentiate BK channels in rat supraoptic neuronal cell bodies and ethanol actually inhibited BK channels in aortic myocytes (Dopico, 2003; Dopico et al., 1999). These different responses to ethanol might result from alternative splicing, alternative tetramer assembly, posttranslational modifications, or association of accessory proteins, phenomena known to alter the functional properties of this channel (Korovkina and England, 2002).

Previously, we used a behavioral assay to show that mutations in *slowpoke*, which eliminate the production of BK channels, also prevent the acquisition of ethanol tolerance (Cowmeadow et al., 2005). We also demonstrated that sedation with the anesthetic benzyl alcohol induces *slowpoke* gene expression and this appears to be linked to benzyl alcohol tolerance (Ghezzi et al., 2004). Here, we ask whether ethanol sedation induces *slowpoke* gene expression and whether an artificial increase in expression can produce a phenocopy of the tolerant state.

METHODS

Flies

Flies were raised on standard cornmeal/molasses/agar medium on a 12/12-hour light–dark cycle (9 AM–9 PM). Newly eclosed flies were collected over a 2-day period and studied when 5 to 7 days old. All experiments were started at approximately the same time of the day (between 12 PM and 6 PM) to minimize the effect of circadian rhythms. Stocks used were Canton S, w^{1118}, and slo^4. Transgenic stocks used were P3 and B52H. Canton S is a wild-type laboratory stock, whereas w^{1118} flies are mutant for an eye pigment gene, but otherwise wild type. The w^{1118} stock was chosen because it was the background stock in which the P3 and B52H lines of transgenic flies were made (Atkinson et al., 1998; Brenner et al., 1996). The slo^4 line of flies are null mutants for the *slowpoke* gene. The P3 stocks contain the P3 transgene in which the neural portion of the *slowpoke* transcriptional control region drives expression of a lacZ reporter gene that encodes the β-galactosidase (β-gal) enzyme (Brenner et al., 1996). The B52H line of transgenic flies carries the B52 transgene in which the HSP70 heat-shock promoter drives expression of a *slowpoke* cDNA in a slo^4 mutant background (Atkinson et al., 1998). No form of sedation (such as CO_2, ether, or cold) was used for any transfer or sorting of the flies. Female flies were used for all assays.

Ethanol Sedation

Flies were sedated with ethanol in an inebriator as described previously (Cowmeadow et al., 2005). Briefly, the inebriator contained 2 treatment chambers. A treatment chamber held 6 standard *Drosophila* vials and, typically, each vial contained 10 flies. Each treatment chamber could receive an ethanol-saturated air stream for sedation of flies or a humidified fresh air stream for mock sedation. The ethanol-saturated air stream was produced by passing humidified air through 2 heated bubblers that contained 100% ethanol heated to 65 °C. The heated ethanol air stream was cooled to room temperature by passage through Teflon tubing.

Benzyl Alcohol Sedation

Flies were sedated with benzyl alcohol in a 30-mL glass vial whose interior had been coated with benzyl alcohol. Vials were coated by adding 200 μL of a 0.4% benzyl alcohol solution in acetone and rotating the vial continuously for 45 minutes at 22 °C. Control vials were made by coating with acetone only. Experimental flies were placed in the benzyl alcohol–coated vials. Control flies were placed in the acetone-coated vials.

Ethanol Tolerance Test

For an ethanol tolerance test, age-matched, female Canton S flies were collected and divided into 12 vials of 10 flies each. Flies were placed in the inebriator: 6 vials in each of the 2 treatment chambers. The control chamber received only fresh, humidified air. The experimental chamber received an ethanol-saturated air stream. The experimental flies were exposed to ethanol until all flies were sedated, about 15 minutes, at which point the ethanol air stream was replaced with the fresh air stream. Flies were scored as sedated when lying on their backs or sides or when "face-down" with their legs splayed out in a nonstandard posture. After experimental flies had recovered from sedation, both control and experimental groups were returned to food vials. Flies were scored as recovered when they had regained postural control. Four or 24 hours later, both groups were returned to the inebriator. However, for this second treatment, both groups were exposed to an ethanol-saturated air stream until all flies were sedated. For the control group, this was the first sedation. For the experimental group, this was the second sedation. Once all flies were sedated, the ethanol air stream was replaced with a fresh air

stream and flies were allowed to recover within the treatment chambers. The number of flies recovered from sedation in each vial was noted once every minute during the entire recovery phase. The percentage of flies recovered was plotted against time.

Cross-tolerance

Age-matched, female w^{1118} flies were collected and divided into 6 vials of 10 to 15 flies each. Flies were placed in the inebriator, 3 vials of flies in each of the 2 treatment chambers. In the experimental group, the flies were sedated with ethanol while animals in the control group were mock sedated (details of the process are given in the previous paragraph). Once the sedated flies had recovered, all flies were returned to food vials. Twenty-four hours later, both groups of flies were sedated with benzyl alcohol as described. Once all experimental flies were sedated they were transferred to clean vials and allowed to recover. Flies were scored as recovered when they had resumed geotactic behavior (climbed side of vial). The number of flies recovered from sedation in each vial was noted once every minute during the entire recovery phase. The percentage of flies recovered was plotted against time. The converse experiment was also performed, in which flies were sedated with benzyl alcohol first, and then 24 hours later, the rate of recovery from ethanol sedation was measured.

Heat-Shock and Ethanol-Resistance Tests

For the first set of experiments, age-matched, female B52H were collected and divided into 6 vials. Three of the vials of flies (experimental) were heat shocked 3 times, at 0, 8, and 16 hours, with each heat shock consisting of 30 minutes in a 37 °C incubator. The other 3 vials of flies (control) remained at room temperature (~21 °C) during this entire period. At 24 hours, all flies were placed in the inebriator and sedated with ethanol as described. This was the first ethanol sedation for both groups of flies. The number of flies recovered from sedation in each vial was noted once every minute during the entire recovery phase. The percentage of flies recovered was plotted against time. The same procedure was repeated for Canton S and P3 flies.

In the second set of heat-shock experiments, B52H flies received only a single heat shock (at 0 hours) and the ethanol sedation took place 6 hours later. Control animals included B52H flies that were not heat shocked and both heat-shocked and non–heat-shocked Canton S flies (wild type).

Behavioral Statistics

For all behavioral tests described above, the log rank test was used to determine whether there was a significant difference between the recovery curves of 2 groups of sedated flies. This test is well suited for the analysis of time to event data in a population (Hosmer et al., 2002). For a population, this test determines significance using all points on the recovery curve rather than on a single time point. It also accounts for animals that fail to recover during the analysis period.

Quantitative Real-Time Polymerase Chain Reaction

Age-matched female w^{1118} flies were placed in the inebriator. Four vials of 7 to 8 flies each were sedated with ethanol, while 4 vials of 7 to 8 flies each were exposed to air only. Total RNA was extracted 6 hours following the start of treatment using the single-step RNA isolation from cultured cells or tissue protocol (Ausubel, 1994). The RNA was treated with RNase-free DNase I (Ambion Inc., Austin, TX) to remove all DNA contamination. RNA was quantified using the RiboGreen® RNA quantitation kit (Molecular Probes Inc., Eugene, OR) according to the manufacturer's instructions.

First-strand cDNA was synthesized from total RNA with gene-specific primers and Superscript II reverse transcriptase (Life Technologies, Gaithersburg, MD). The cDNA was amplified by quantitative real-time PCR in an ABI Prism 7700 sequence detection system (Applied Biosystems, Foster City, CA) in the presence of gene-specific dual-labeled single-stranded probes. Polymerase chain reaction was performed using the TaqMan probes and the TaqMan Universal PCR Master mix (Applied Biosystems). Each PCR was performed in triplicate, and the yields thereof were expressed as an average. Messenger RNA abundance was quantified using the standard curve method. Significance was calculated using Student's *t* test.

The primers and probes used were as follows: cyclophilin I upper primer 5′-accaaccacaacggcactg-3′; cyclophilin I lower primer 5′-tgcttcagctcgaagttctcatc-3′; cyclophilin I probe 5′-(FAM)-cggcaagtccatctacggcaacaagtt-(TAMRA)-3′; *slowpoke* exon C1 upper primer 5′-aaacaaagctaaataagttgtgaaagga-3′; *slowpoke* exon C1 lower primer 5′-gatagttgttcgttcttttgaatttga-3′; *slowpoke* exon C1 probe 5′-(FAM)-agaaactgcgcttagtcacactgctcatgt-(TAMRA)-3′; *slowpoke* exon C2 upper primer 5′-gctatttataatagacgggccaagtt-3′; *slowpoke* exon C2 lower primer 5′-ggaaatccgaaagatacgaatgat-3′; and *slowpoke* exon C2 probe 5′-(FAM)-ctcagcctcacaatgcgaaacgga-(TAMRA)-3′.

β-Galactosidase and Protein Assays

Age-matched, female P3 flies were collected and divided into 2 groups of 150 flies each. Each group was placed in the inebriator in a treatment chamber consisting of a 1-L polypropylene bottle. One group of flies, the control group, was exposed to a fresh air stream. The other group, experimental, was exposed to an ethanol-saturated air stream until all flies were sedated, at which point they were switched to a fresh air stream. After all ethanol-treated flies had recovered, the experimental and control groups were transferred to food vials, 10 flies per vial. At various time points following the initial ethanol treatment, 3 vials of ethanol-treated and 3 vials of control P3 flies were assayed for both β-gal activity and total protein levels.

Whole-fly lysate was made from a group of 10 flies by homogenizing them in 1 mL of assay buffer (50 mM KPO_4, pH 7.5/1 mM $MgCl_2$) and then centrifuging to remove debris. β-Galactosidase levels in the lysate were measured using the BIO-RAD FluorAce β-galactosidase assay kit according to manufacturer's instructions (Bio-Rad Laboratories, Hercules, CA). The protein assay was performed on the lysate using the Bio-Rad dye concentrate according to the manufacturer's instructions (Bio-Rad Laboratories). For each group of ten flies, β-gal levels were normalized against total protein levels. Each data point is the average of 3 groups of ten flies each. Wild-type flies have a small level of endogenous β-gal activity. To account for this, 3 vials of 10 flies each of matched Canton S flies were also assayed for β-gal activity and protein levels and these values were subtracted from that of P3. Significance was calculated using Student's *t* test.

RESULTS

Ethanol Increases Neural slowpoke mRNA Levels

Previous studies have shown that solvent anesthetics cause a small induction in *slowpoke* gene expression (Ghezzi et al., 2004). We wished to determine whether sedation with ethanol produced a similar response. Therefore, flies were sedated with ethanol and their *slowpoke* mRNA levels were measured relative to mock-treated animals.

Six hours after ethanol sedation, levels of neurally expressed *slowpoke* mRNA, as measured by quantitative

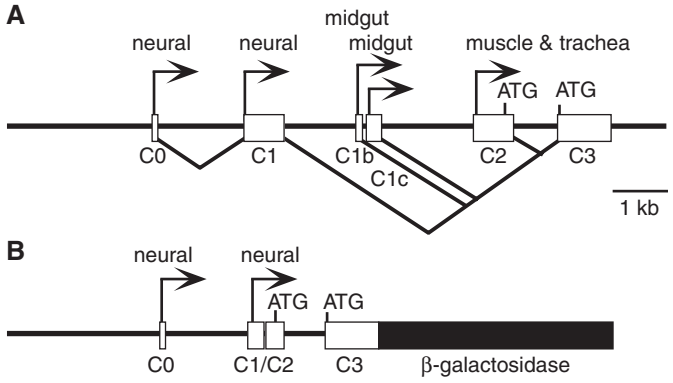

Fig. 1. Transcriptional control region of the *slowpoke* gene and the P3 *slowpoke* reporter gene. (**A**) Transcriptional control region of the *slowpoke* gene. The arrows identify the position of transcriptional promoters mapped by 5′-RACE (Bohm et al., 2000; Brenner et al., 1996). The tissue specificity of the promoters is indicated above the arrows. The open boxes represent exons and the diagonal connecting lines denote the splicing pattern of the mRNA. Exon names (C0, C1, C1b, C1c, C2, and C3) are shown below the open boxes. These names are also used to refer to the transcriptional promoter that expresses each exon. ATG represents putative translation start sites. The bulk of the coding sequence is not shown. (**B**) The P3 *slowpoke* reporter transgene contains only the neural promoters and other sequences required to reproduce the *slowpoke* neural expression pattern (Brenner et al., 1996). The black box represents the lacZ reporter gene that encodes β-galactosidase.

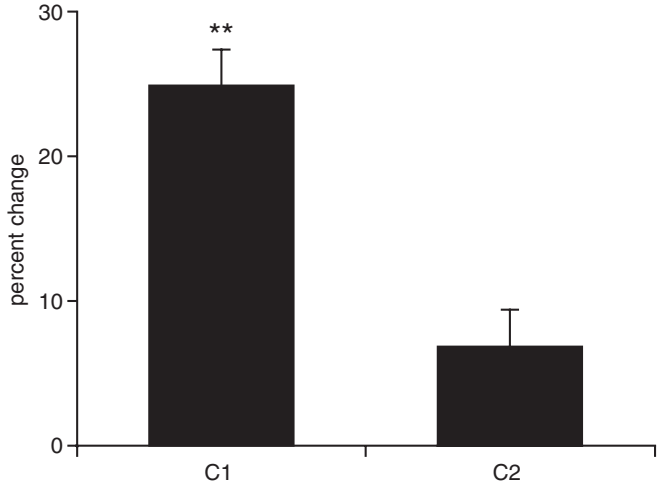

Fig. 2. Ethanol sedation induces expression from the *slowpoke* neural promoters but not the muscle/tracheal cell promoter. The change in the abundance of *slowpoke* mRNA 6 hours after ethanol sedation was determined by quantitative real-time RT-PCR. Both of the neural promoters produce mRNA containing exon C1 and lacking exon C2. The muscle/tracheal cell promoter produces mRNA containing exon C2 and lacking exon C1. Therefore, mRNA expressed from the neural promoters can be quantified using primers that specifically recognize exon C1, whereas mRNA expressed from the muscle promoters can be measured using primers that specifically recognize exon C2. The abundance of *slowpoke* mRNA was normalized against the *cyclophilin* 1 mRNA internal control. Levels of neurally expressed (C1), but not muscle/tracheal cell expressed (C2), *slowpoke* mRNA were significantly increased 6 hours after ethanol sedation (error bars are standard error of the mean, $n = 3$; $^{**}p < 0.001$).

real-time PCR using primers specific for exon C1, were significantly higher in ethanol-treated animals compared with animals exposed only to air (Figs. 1A and 2). Conversely, using primers specific for exon C2, levels of muscle-specific mRNA did not show a significant change in ethanol-treated animals (Figs. 1A and 2). As an internal control, we also measured the relative abundance of mRNA from the cyclophilin gene. Cyclophilin is a cell cycle gene and its mRNA has been used by others as an internal control for changes in gene expression following ethanol sedation (Follesa et al., 2004; Jarvelainen et al., 1999). We have also observed that cyclophilin mRNA abundance is not affected by sedation with other solvents such as benzyl alcohol (Ghezzi et al., 2004). The abundance of *slowpoke* mRNA was normalized against that of cyclophilin.

Increase in slowpoke mRNA Is a Transcriptional Response

The increase in *slowpoke* mRNA following ethanol sedation might arise from increased transcription initiation, increased mRNA stability, or a combination of both. To determine whether sedation alters the rate of transcription initiation, we measured whether ethanol sedation affects the expression of a different gene driven by the same transcriptional control region. The stability of an mRNA is largely determined by its 3′ end, so it is unlikely that events that alter the stability of the *slowpoke* mRNA would also alter the stability of another mRNA.

For these experiments, we used a transgene called P3, which contains the neural portion of the *slowpoke* transcriptional control region, but not elements responsible for expression in other tissues. In P3, this control region drives expression of the β-gal reporter gene, which is terminated by the SV40 polyadenylation signal (Fig. 1B) (Brenner et al., 1996). We chose this transgene because previous experiments showed that flies lacking only neural portions of the *slowpoke* transcriptional control region were unable to acquire ethanol tolerance (Cowmeadow et al., 2005).

Ethanol-treated and mock-treated P3 flies were assayed for both β-gal activity and total protein levels. β-Galactosidase levels were then normalized against protein levels. We did not see a significant change in β-gal specific activity 6 hours after treatment (data not shown), even though we did observe an increase in *slowpoke* mRNA at this time. A time course revealed that induction of β-gal from the transgene was not apparent until 18 hours and peaked at 21 hours (Fig. 3).

Artificially Inducing slowpoke Leads to Ethanol Resistance

We have shown that flies increase their expression of *slowpoke* 6 hours following ethanol sedation. We have previously shown that wild-type flies, but not *slowpoke* mutant flies, will gain tolerance to ethanol within this same time frame (Cowmeadow et al., 2005). We next wished to determine whether an artificial increase in *slowpoke* expression would cause flies exposed to ethanol for the

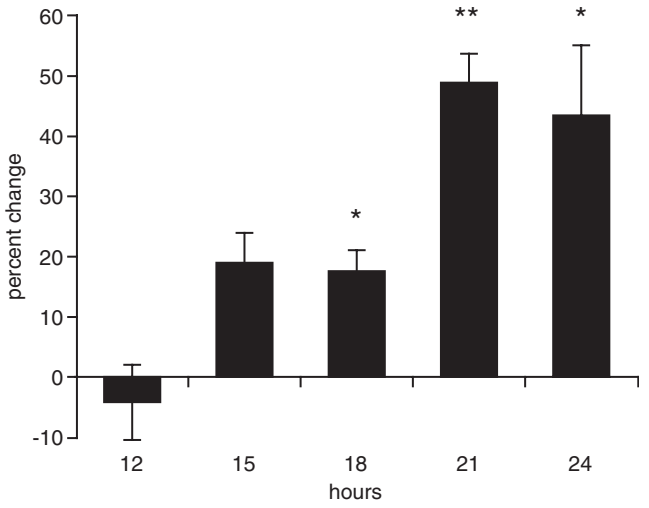

Fig. 3. Reporter gene expression following ethanol sedation. Expression of β-galactosidase from the P3 reporter transgene was measured at various time points after ethanol treatment ($n = 3$ for each time point). The P3 transgene contains only the *slowpoke* neural promoters. Expression of β-galactosidase in P3 flies increases over time, peaking at approximately 21 hours. Error bars are standard error of the mean. $^{*}p<0.05$, $^{**}p<0.01$.

first time to behave as if they had already acquired tolerance. To answer this question, we used a line of transgenic flies called B52H. These flies carry a transgene in which the HSP70 heat-shock promoter drives expression of a *slowpoke* cDNA in a *slowpoke* mutant background (Atkinson et al., 1998). These flies do have some basal level of *slowpoke* expression because of leakiness of the heat-shock promoter. However, when these flies are heat-shocked, their *slowpoke* expression increases 2- to 3-fold (Ghezzi et al., 2004).

To test the effect of increased *slowpoke* on ethanol resistance, B52H flies were divided into 2 groups. The experimental group was heat shocked to induce *slowpoke* expression while the uninduced, control group remained at room temperature. In the first set of experiments, the heat-shock group received three 30-minute 37 °C heat shocks separated by 8 hours. Twenty-four hours after the beginning of the first heat-shock, both groups were sedated with ethanol and their recovery times were noted. The heat-shocked B52H flies recovered from sedation significantly faster than their non–heat-shocked counterparts (Fig. 4A). This response is very similar to that seen in flies that had previously been sedated with ethanol (Fig. 4B). When the same experiment was performed on wild-type flies or on a different line of transgenic flies, no difference in recovery times was seen between the heat-shocked and non–heat-shocked groups (Figs. 4C and 4D).

Next, the heat-shock protocol was modified to more closely resemble a bout of ethanol sedation. In these experiments, the experimental group of B52H flies was heat shocked once for 30 minutes at 37 °C, while the control group of B52H flies remained at room temperature. Six hours later, both groups of flies were sedated with ethanol and their recovery times were noted. The heat-shocked group recovered from sedation significantly faster than the non–heat-shocked group, demonstrating that a single bout of *slowpoke* induction is sufficient to induce ethanol resistance (Fig. 5A). When the same experiment was performed on wild-type flies, no significant difference in recovery times was observed (Fig. 5B).

Cross-tolerance Between Ethanol and Benzyl Alcohol

We have observed similarities in the response of flies to ethanol and to benzyl alcohol (Cowmeadow et al., 2005; Ghezzi et al., 2004), which led us to believe that tolerance to these drugs are mechanistically related. To confirm this idea, we asked whether prior sedation with one drug produces cross-tolerance to the other drug. Twenty-four hours after flies had been sedated with benzyl alcohol or mock sedated, they were sedated with ethanol and their recovery times were noted. We chose to assay for cross-tolerance 24 hours after sedation because all previous tests for benzyl alcohol tolerance were performed 24 hours after sedation (Ghezzi et al., 2004) while ethanol tolerance has been shown to persist for at least 7 days (Cowmeadow et al., 2005). Flies previously sedated with benzyl alcohol recovered significantly faster from ethanol sedation than their mock-treated counterparts (Fig. 6A). Similarly, flies previously sedated with ethanol recovered more rapidly from benzyl alcohol sedation than the mock-treated controls (Fig. 6B).

DISCUSSION

We are using a *Drosophila* model to study the molecular mechanism of drug tolerance. In *Drosophila*, rapid tolerance to ethanol or the anesthetic benzyl alcohol requires a functional *slowpoke* gene, suggesting that ethanol tolerance and benzyl alcohol tolerance share common molecular mechanisms (Cowmeadow et al., 2005; Ghezzi et al., 2004). Further, tolerance to the anesthetic benzyl alcohol has been linked to a sedation-induced increase in *slowpoke* gene expression (Ghezzi et al., 2004). Here, we wished to determine whether ethanol sedation induces *slowpoke* gene expression and whether the induction of *slowpoke* gene expression, alone, was sufficient to produce a phenotype that mimics ethanol tolerance.

We observed that a brief ethanol sedation caused an increase in *slowpoke* mRNA abundance in the nervous system. This transcriptional response is notably specific. Real-time PCR primers targeted against neural and muscle-specific transcripts showed that the mRNA produced by the neural promoters increased in abundance, while mRNA produced from the muscle/trachea promoter was unchanged. This illustrates that regulation of the *slowpoke* gene is highly granular and that environmental cues can differentially modulate expression in a tissue-specific manner.

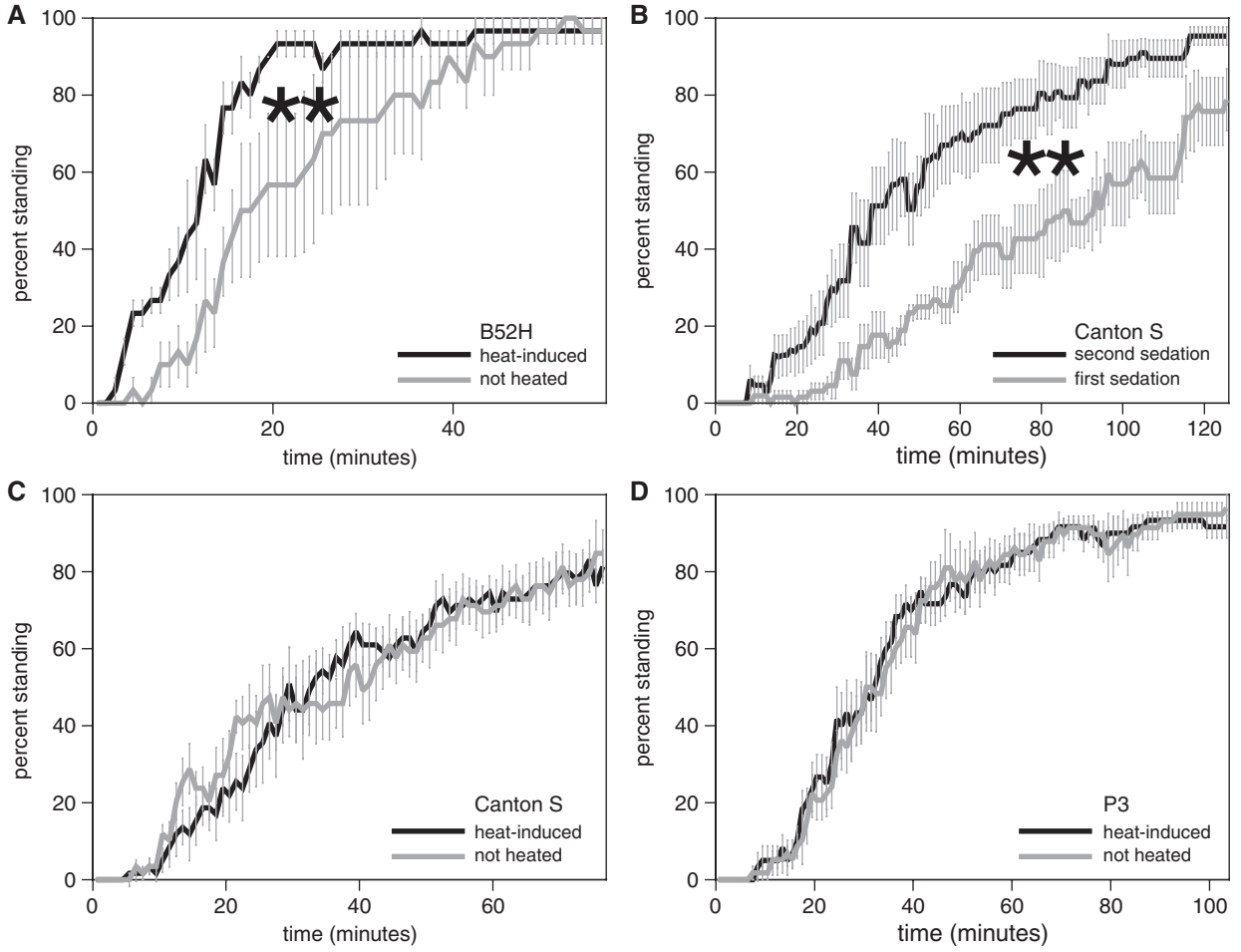

Fig. 4. Chronic induction of a *slowpoke* transgene causes ethanol resistance. (**A**) The B52H transgene expresses a *slowpoke* cDNA from a heat-inducible transcriptional promoter (Atkinson et al., 1998). Three 30-minute bouts of heat shock separated by 8 hours were used to induce the transgene. Eight hours after the last induction, flies showed ethanol resistance ($n = 30$; $p < 0.01$). (**B**) Wild-type Canton S flies manifest tolerance to ethanol 24 hours after a single ethanol sedation ($n = 64$; $p < 0.001$). (**C**) The heat-shock paradigm does not cause wild-type Canton S flies to acquire resistance ($n = 59$. $p < 1.0$). (**D**) Inducible resistance is not a side effect of the transgene vector. P3 flies, which carry the same transgenic vector as B52H, do not acquire resistance after the heat-shock treatment ($n = 60$, $p < 0.7$). Significant difference between the curves was determined using the log rank test; however, the error bars are standard error of the mean for each data point. $^{**}p < 0.01$.

The sedation-induced increase in *slowpoke* expression was also seen with the P3 transgene. In P3, the neural portion of the *slowpoke* transcriptional control region drives expression of the β-Gal reporter gene. Expression from P3 was also enhanced following ethanol sedation. We did not observe increased P3 expression until 18 hours after sedation, even though induction from the endogenous *slowpoke* gene was apparent 6 hours after sedation. These differences in induction rate between the endogenous gene and a transgenic reporter gene are not surprising. The chromosomal position of the P3 transgene is expected to have some effect on the kinetics of its activation. Alternatively, the time differential may be due to the lag between initiation of mRNA induction and translation of detectable levels of β-Gal. The salient point is that P3 and *slowpoke* expression were both ethanol-inducible.

The alteration in *slowpoke* transcription because of ethanol sedation indicates that there must be regulatory elements present in the *slowpoke* transcriptional control region that mediate the response. More specifically, these elements must be in the 5 kb of transcriptional control region carried in the P3 transgene. Such elements might not respond directly to ethanol itself but to a change in neural excitability caused by ethanol. Ghezzi et al. (2004) have shown that even cold and CO_2 sedation, treatments that are predicted to reduce neural excitability, also increase *slowpoke* expression. Therefore, we suspect that the increase in *slowpoke* expression is a compensatory response to the reduced excitability of the nervous system.

A transcription factor that may be involved in regulating *slowpoke* expression in response to ethanol is cyclic-AMP response element binding protein (CREB). Changes in the activity of this transcription factor have been implicated as underlying some of the long-lasting changes in neural gene expression following ethanol exposure (Nestler, 2001).

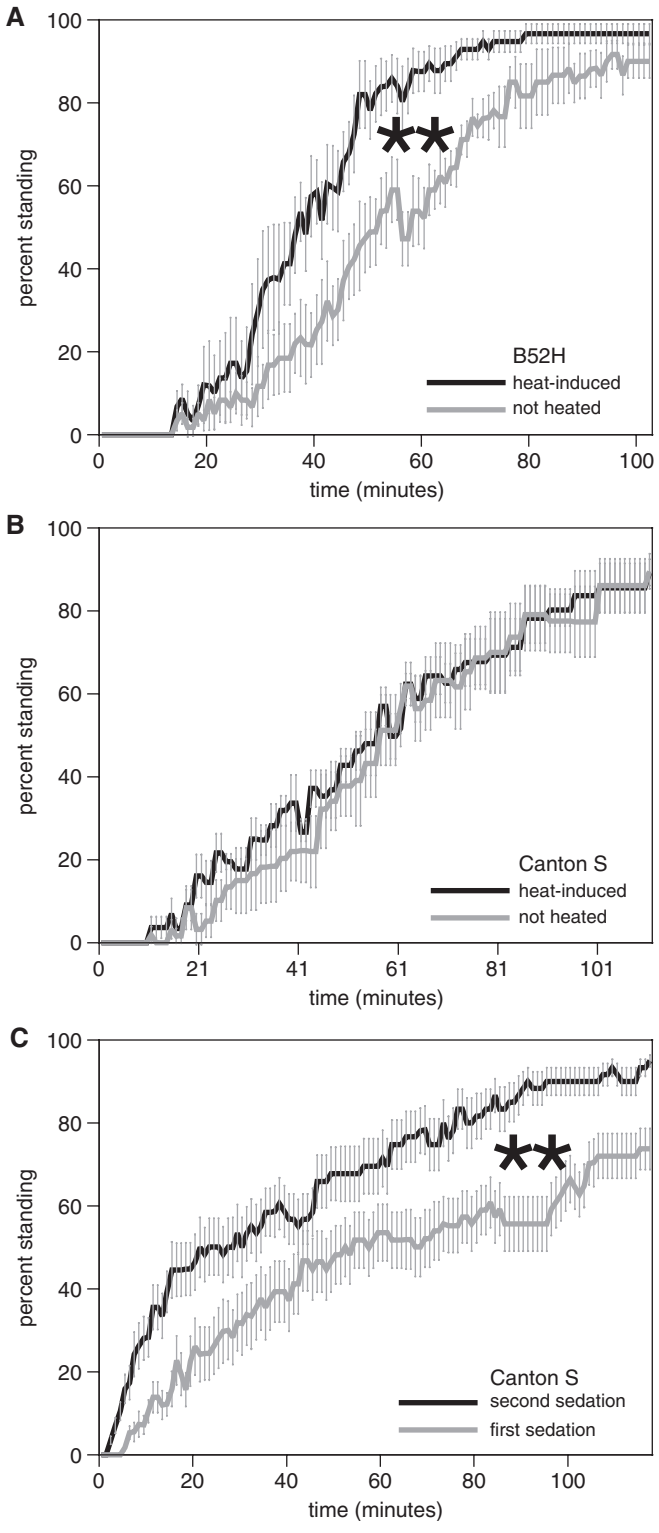

Fig. 5. A single heat pulse induction of the *slowpoke* transgene is sufficient to cause ethanol resistance. (**A**) The B52H transgene expresses a *slowpoke* cDNA from a heat-inducible transcriptional promoter (Atkinson et al., 1998). Six hours after a single 30-minute heat induction, ethanol resistance is apparent ($n = 56$; $p < 0.01$). (**B**) Wild-type Canton S flies do not show ethanol resistance 6 hours after a single 30-minute heat treatment ($n = 56$; $p < 0.9$). (**C**) Wild-type Canton S flies manifest tolerance to ethanol 4 hours after a single ethanol sedation ($n = 56$; $p < 0.001$). Significant difference between the curves was determined using the log rank test; however, the error bars are standard error of the mean for each datapoint. $^{**}p < 0.01$.

DNA-binding sites for CREB have been found in many genes expressed in the nervous system. Gene regulation by CREB has been implicated in learning, memory and cellular responses to drugs of abuse (Nestler, 2004). Cyclic-AMP response element binding protein activity is regulated by phosphorylation, and in mammals acute ethanol treatment has been shown to increase levels of phosphorylated CREB, while chronic ethanol treatment decreases phosphorylated CREB levels in several brain structures (Li et al., 2003; Misra and Pandey, 2003; Misra et al., 2001; Pandey et al., 2001, 2003; Yang et al., 1998, 2003). The *Drosophila slowpoke* gene also contains putative binding sites for the CREB transcription factor (data not shown), and thus in *Drosophila*, CREB may also be involved in the ethanol response.

We have previously shown that mutant flies lacking *slowpoke* expression cannot acquire rapid tolerance to ethanol (Cowmeadow et al., 2005), and now we show that the acquisition of tolerance in wild-type flies is coincident with an increase in *slowpoke* expression. These observations are consistent with the hypothesis that increased *slowpoke* expression is the cause of tolerance. If this were the case, then one would expect that increasing *slowpoke* expression without exposure to ethanol would cause flies to behave as if they had already acquired tolerance. This is exactly what we observed. We used the B52H line of transgenic flies in which a heat-shock promoter drives expression of a *slowpoke* cDNA. Heat shock causes a substantial increase in *slowpoke* expression over basal levels (Ghezzi et al., 2004). Heat-shocked B52H flies recovered from their first ethanol sedation more rapidly than non–heat-shocked B52H flies. This was not due to a heat-shock response, or a nonspecific effect of the transgene vector, as no difference was seen in ethanol recovery times when the same experiment was performed on both wild-type flies and another line of transgenic flies. While we do not believe that a single gene could be responsible for all aspects of ethanol tolerance, this certainly demonstrates that the *slowpoke* gene plays a pivotal role in this phenomenon.

If, as we suspect, the increase in *slowpoke* expression is a compensatory response to the ethanol-induced inhibition of neural excitability, then the BK channel must play an excitatory role in the relevant cells or circuits. The BK channel could be playing an excitatory role either by decreasing the excitability of inhibitory circuits or by increasing the excitability of excitatory circuits. Unfortunately, in *Drosophila*, we do not have a detailed map of excitatory and inhibitory circuits, nor do we know where the induction of *slowpoke* occurs.

However, knowledge of the identity of the cells or circuits in which *slowpoke* expression is induced would not necessarily simplify matters as BK channels differ in both their intrinsic properties and in their effects on excitability. BK channels can have many different electrophysiological properties because of factors such as

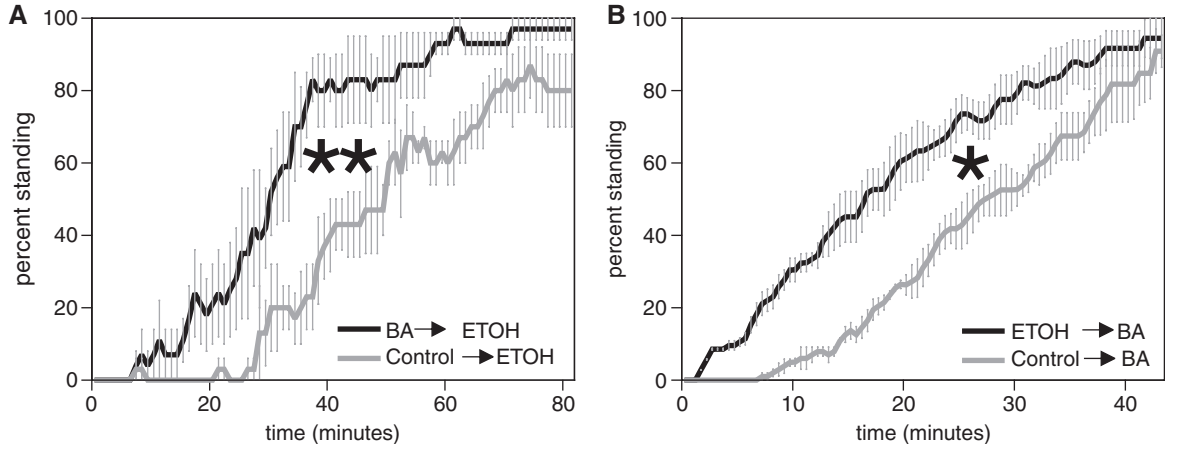

Fig. 6. Cross-tolerance between ethanol and benzyl alcohol. Cross tolerance was demonstrated (**A**) in flies sedated with benzyl alcohol and then later tested for tolerance to ethanol ($n = 30$; $p < 0.001$) or (**B**) in flies sedated with ethanol and later tested for tolerance to benzyl alcohol ($n = 45$; $p < 0.03$). Tolerance tests were performed 24 hours after the first sedation. Significant difference between the curves was determined using the log rank test; however the error bars are standard error of the mean for each datapoint. $^{**}p < 0.01$, $^{*}p < 0.05$.

alternative splicing, posttranslational modifications, heterotetramer assembly, and association of accessory proteins (Korovkina and England, 2002). The differences in intrinsic channel properties may help explain the contradictory roles played by BK channels in neural excitability. In most cases, BK channel activity is inversely correlated with cellular excitability. That is, the increased potassium efflux caused by enhanced BK channel activity depresses neural excitability (Gribkoff et al., 2001; Orio et al., 2002; Sun and Dale, 1998). However, apparent contradictions have also been observed in which BK channel activity is directly correlated with cellular excitability. In these contradictory examples, the outward potassium current through the BK channel is still proposed to reduce the instantaneous excitability of the cell, and by doing so, prevent the activation of other channels whose action would lead to a long-term reduction in neuronal firing (Lovell and McCobb, 2001; Pattillo et al., 2001; Van Goor et al., 2001; Warbington et al., 1996; Xu et al., 2005).

A role for *slowpoke* in the response to ethanol seems to be evolutionarily conserved. Davies et al. (2003) have shown that *C. elegans* mutant for *slowpoke* show resistance to ethanol's effects on locomotion and that hyperactivation of *slowpoke* mimics intoxication. In the rat hypothalamic-neurohypophysial system, which is responsible for the release of oxytocin and vasopressin, ethanol tolerance at the molecular level involves a reduction in BK channel potentiation and density (Pietrzykowski et al., 2004). While these results are not consistent with our observations in flies, they demonstrate that, in these very different animals, the *slowpoke* gene plays a significant role in the ethanol response.

We have clearly shown a link between ethanol sedation, ethanol tolerance, and induction of *slowpoke* expression. Recently, we reported similar observations for sedation with the anesthetic benzyl alcohol (Ghezzi et al., 2004). In addition, here we show that sedation with ethanol or benzyl alcohol causes cross-tolerance to the other drug. We interpret this to mean that tolerance to both drugs occurs via the same mechanism. We propose that the change in *slowpoke* expression is not a response to a particular drug, but is a response to depressed neural activity and that the increased *slowpoke* expression counters this depression and thereby either causes or triggers tolerance.

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