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A DNA element in the *slo* gene modulates ethanol tolerance

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Abstract

In *Drosophila*, the *slo* gene encodes BK-type Ca^{2+} -activated K^+ channels and is involved in producing rapid functional tolerance to sedation with ethanol. *Drosophila* are ideal for the study of functional ethanol tolerance because the adult does not acquire metabolic ethanol tolerance (Scholz, Ramond, Singh, & Heberlein, 2000). It has been shown that mutations in *slo* block the capacity to acquire tolerance, that sedation with ethanol vapor induces *slo* gene expression in the nervous system, and that transgenic induction of *slo* can phenocopy tolerance (Cowmeadow, Krishnan, & Atkinson, 2005; Cowmeadow et al., 2006). Here we use ethanol-induced histone acetylation to map a DNA regulatory element in the *slo* transcriptional control region and functionally test the element for a role in producing ethanol tolerance. Histone acetylation is commonly associated with activating transcription factors. We used the chromatin-immunoprecipitation assay to map histone acetylation changes following ethanol sedation to identify an ethanol-responsive DNA element. Ethanol sedation induced an increase in histone acetylation over a 60-n DNA element called 6b, which is situated between the two ethanol-responsive neural promoters of the *slo* gene. Removal of the 6b element from the endogenous *slo* gene affected the production of functional ethanol tolerance as assayed in an ethanol-vapor recovery from sedation assay. Removal of element 6b extended the period of functional ethanol tolerance from ~10 days to more than 21 days after a single ethanol-vapor sedation. This study demonstrates that mapping the position of ethanol-induced histone acetylation is an effective way to identify DNA regulatory elements that help to mediate the response of a gene to ethanol. Using this approach, we identified a DNA element, which is conserved among *Drosophila* species, and which is important for producing a behaviorally relevant ethanol response.

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Keywords

Drosophila; ethanol tolerance; epigenetics; histone acetylation; BK channel

Introduction

Alcoholism is a disease of altered behavior produced by ethanol-induced changes in brain function. In the United States, almost 4% of the population meet the criteria for alcoholism or alcohol addiction, and the annual costs of alcohol-related problems are estimated to exceed 223 billion dollars (Bouchery, Harwood, Sacks, Simon, & Brewer, 2011; Grant et al., 2004). Unfortunately, the long-term success rate of treatment for alcoholism is dismal. Two-thirds of individuals have bouts of heavy drinking in their first year of treatment and overall, the three-year recidivism rate among remitted individuals is ~25% (Dawson, Goldstein, & Grant, 2007; Miller, Walters, & Bennett, 2001; Tuithof, Ten Have, van den Brink, Vollebergh, & de Graaf, 2013). A major contributor to alcohol addiction is thought to be the collective neural adaptations produced by repeated ethanol exposure. These adaptations may contribute to the addictive process directly, and indirectly because of the negative effects (withdrawal symptoms) they produce after alcohol clearance (Koob et al., 2013).

The earliest neural adaptation to ethanol is functional tolerance. Functional tolerance is defined as a reduced response to a given concentration of ethanol. It is distinct from metabolic tolerance, which is the product of an increase in the rate of ethanol clearance. The same adaptations that produce functional tolerance have been linked to physiological dependence and the accompanying symptoms of ethanol withdrawal (Ghezzi & Atkinson, 2011; Koob & Le Moal, 2006; Martin, 1968). However, the correlation between the capacity to acquire tolerance and increased drinking behavior is complex, and may depend both on the way tolerance was induced and the way it was measured (Crabbe et al., 2012; Fritz, Grahame, & Boehm, 2013; Matson, Kasten, Boehm, & Grahame, 2014). Nevertheless, it is probable that the later adaptations, which produce the addicted state, bear some resemblance to or are built upon the early adaptations that underlie tolerance.

In *Drosophila*, BK-type Ca^{2+} -activated K^{+} channels, encoded by *slo*, are involved in producing rapid tolerance to sedation with ethanol – it has been shown that mutations in *slo* block the capacity to acquire tolerance, sedation with ethanol vapor induces *slo* gene expression in the nervous system, and transgenic activation of *slo* expression can phenocopy tolerance (Cowmeadow et al., 2005, 2006). The role of BK channels in producing ethanol tolerance is not unique to flies and has also been demonstrated in mammals (Treisman & Martin, 2009).

We have been interested in how ethanol alters gene expression in ways that contribute to ethanol adaptation. Toward this end we have focused on ethanol-induced transcriptional regulation of the *slo* gene. A common feature of transcription factors that stimulate gene expression is that they recruit histone acetyl transferases (HATs) to the region. The addition of acetyl groups to the histones modifies the structure of the chromatin to make the DNA more accessible. In addition, acetylated histones serve as binding sites that attract the basal

transcription factors required for transcription initiation (Dhalluin et al., 1999; Lee, Hayes, Pruss, & Wolffe, 1993). Here we show that mapping the position of ethanol-induced histone acetylation is a useful way to identify the DNA regulatory element that helps to mediate ethanol-related behaviors. A 60-n conserved DNA element was identified as the putative site of ethanol-induced transcription factor activity, based on its histone acetylation profile. Removal of this element from the endogenous gene produced a profound increase in the duration of ethanol tolerance.

Methods

Drosophila stocks

Our wild-type stock is the Canton S line obtained from the Bloomington *Drosophila* Stock Center. The construction of the *slo*^{6b} stock has been described in Li, Ghezzi, Pohl, Bohm, and Atkinson (2013). The construction of *slo*^{55b} control stock has been described in Li et al. (2015). Neither of these stocks contain any other genetic marks. Both stocks have been backcrossed six times into the Canton S genetic background.

Functional tolerance assay

Animals were tested for their capacity to acquire functional ethanol tolerance essentially as described by Krishnan, Al-Hasan, Pohl, Ghezzi, and Atkinson (2012), except that the time of the second sedation was varied from 1 to 28 days. Briefly, 5–7-day-old mated female flies (products of the same culture bottles) were placed into 12 glass vials (10 flies per vial) into which was pumped a humidified, ethanol-saturated vapor stream (experimental) or humidified air (control, mock sedation). Using a single sex reduces variability in the behavioral response. Flies were exposed until sedated. Flies were scored as sedated when they were lying on their backs or sides or if they remained upright with their legs splayed in a non-standing position. Once sedated, the animals were switched over to a fresh-air stream and allowed to recover. After the experimental flies had recovered, both the experimental and control animals were returned to food vials for 1 to 28 days. Flies were changed to new vials on a weekly basis. After this time interval, both experimental and control animals were sedated in an ethanol airstream (as described above). For the experimental animals, this was their second sedation; for the control animals this was their first sedation. After the flies were sedated, the ethanol airstream was replaced with a fresh-air airstream. The number of flies recovered from sedation in each vial was noted in 2-min intervals. Recovery was visually determined as a return of postural control. Animals were said to display tolerance if the recovery curve of second-time sedated animals was more rapid than first-time sedated animals. The separation of the two curves was statistically evaluated using the Log-rank analysis.

Chromatin immunoprecipitation assay

The chromatin immunoprecipitation assay was performed on chromatin isolated from *Drosophila* heads as described in Li et al. (2013). Ethanol-treated flies were prepared as described for the Functional Tolerance Assay (above), except that 500 animals were exposed in a 1-liter container. To determine the time course of histone H4 acetylation, chromatin was prepared from about 500 fly heads, 0.5, 6, 24, 48, and 96 h after ethanol sedation. Real-time

PCR was used to assay the conserved DNA elements C0, 4b, 6b, C1, cre1, s2, 55b, C2, and cre2 using the primers C0 (5'-ATCGAACGAAGCGTCCAG-3', 5'-CGACGCGCTCAAACG-3'), 4b (5'-GACCCGATGATAAAGTCGATGT-3', 5'-GCCAGTGACTGACTGACACACA-3'), 6b (5'-CCAGCAGCAATTGTGAGAAA-3', 5'-CGAAGCAGACTTGAAAGCAA-3'), C1 (5'-ACAAACCAAAACGCACAATG-3', 5'-AATGGATGAAGGACTGGGAGT-3'), cre1 (5'-GATGGGAAAGCGAAAAGACAT-3', 5'-CATGTCCGTCAAAGCGAAAC-3'), s2 (5'-CATTGCTATCCCTTCCCATC-3', 5'-ATGCAATGAAGCGAAGAACC-3'), 55b (5'-ACCCAATTGAATTCGCCTTGTCTT-3', 5'-CCCACTCTCCGGCCATCTCT-3'), C2 (5'-GCACTCGACTGCACTTGAAC-3', 5'-AATGAAAAAGTTCTCTCTGTGCAT-3'), and cre2 (5'-TGGATTGCGACCGAGTGTCT-3', 5'-ATCAATACGATAACTGGCGGAAACA-3'). All amplicons have differences in standard curve amplification slopes of less than 0.1. Amplifications were run in duplicate. Melting curves were used to detect nonspecific amplification. The relative amount of the acetylated H4 histone was calculated by the $2^{-(Ct_{IP} - Ct_{Input})}$ method. Fold enrichment over control is equal to $2^{(Ct_{Input} - Ct_{IP})_{experimental} / (Ct_{Input} - Ct_{IP})_{control}}$. All data were normalized to Cyp1 using the primers 5'-TCTGCGTATGTGTGGCTCAT-3' and 5'-TACAGAACTCGCGCATTAC-3'. Chromatin immunoprecipitation assays were performed at least three times with independent chromatin samples and the mean and SEM were calculated. Statistical significance was determined by one-way ANOVA with Dunnett's comparison.

Results

In *Drosophila*, ethanol and benzyl alcohol induce mutual cross tolerance, indicating that tolerance to these drugs arises from an overlapping mechanism (Cowmeadow et al., 2006; Ghezzi et al., 2013). Both ethanol and benzyl alcohol induce expression from the *slo* gene, and this induction contributes substantially to the production of behavioral tolerance to these drugs (Cowmeadow et al., 2006; Ghezzi, Al-Hasan, Larios, Bohm, & Atkinson, 2004). The transcriptional control region of the *slo* BK-type Ca^{2+} -activated K^{+} channel includes five tissue-specific core promoters (Fig. 1, top). Between these core promoters lie small blocks of sequence that are highly conserved in all sequenced *Drosophila* species. These sequences are thought to be regulatory elements that modulate the expression pattern of the neighboring promoters (Bohm, Wang, Brenner, & Atkinson, 2000; Wang, Krishnan, Ghezzi, Yin, & Atkinson, 2007). Activating transcription factors commonly promote local histone acetylation. Therefore, in Li et al. (2013), the locations of benzyl alcohol-induced histone acetylation changes were used to map a DNA regulatory element in the *slo* gene that is important for the manifestation of behavioral tolerance. This 60-n element is called 6b. Deletion of element 6b caused a striking increase in the duration of benzyl alcohol tolerance. Based on these observations using benzyl alcohol, we asked whether ethanol also induces histone acetylation over the *slo* 6b element and whether deletion of this 60-n element increased the duration of ethanol tolerance.

To determine the time course of histone H4 acetylation following ethanol sedation, 5–7-day-old female flies were sedated with ethanol vapor, allowed to recover, and stored with food for 30 min, 4 h, 6 h, 24 h, 48 h, or 96 h. Chromatin was purified from fly heads and subjected to a chromatin immunoprecipitation assay using an antibody that recognized all

forms of acetylated histone H4. We observed a prominent acetylation spike over element 6b, 6 h after sedation that was still detectable at 48 h post-sedation. This increase in histone acetylation persisted until 48 h after ethanol sedation (Fig. 1). This strong targeting of the histone H4 acetylation to the 6b element after ethanol sedation suggests that it may play an important role in a *slo*-mediated response to ethanol.

The *slo*^{6b} mutant stock was described in Li et al. (2013). It is the product of homologous recombination mutagenesis (Gong & Golic, 2003) in which the 60-n 6b element of the endogenous *slo* gene was precisely replaced with a 70-n loxP site. This mutant allele is called *slo*^{6b}. The mutant animal was backcrossed 6-fold into our standard Canton S wild-type background before testing. Behavioral analysis of this stock showed that homozygous animals displayed normal locomotor activity, climbing, capacity for flight, longevity, and circadian rhythms (Li et al., 2013). However, in Li et al. (2013), the *slo*^{6b} mutation is shown to specifically alter the time course of functional tolerance to the organic solvent inhalant benzyl alcohol. In particular, the *slo*^{6b} mutation substantially increased the duration of functional benzyl alcohol tolerance.

To determine whether the *slo*^{6b} mutation had a similar behavioral effect on functional ethanol tolerance, we compared the rate at which flies recovered from their first and their second ethanol sedations. The time of the second sedation was varied from 1 to 28 days so the perdurance of tolerance could be determined (Fig. 2A). In these assays, the animals are functionally tolerant if the recovery curve from the second sedation is left-shifted with regard to the recovery curve from the first sedation (i.e., the tolerance population recovers more rapidly from sedation). In this assay, tolerance cannot be observed during the sedation phase because the concentrated dose of ethanol vapor produces rapid sedation.

Wild-type flies show functional ethanol tolerance at 1 day, 4 days, and 10 days after the first ethanol sedation (Fig. 2B, column 1). By 14 days after ethanol sedation, tolerance has disappeared. However, mutants homozygous for the *slo*^{6b} mutation show tolerance that lasts at least 21 days after ethanol sedation (Fig. 2B, column 2). This unusual phenotype does not appear to be caused by the inserted loxP site because insertion of loxP into a neighboring site (*slo*^{55b} mutant) did not produce prolonged tolerance (Fig. 2B, column 3). In addition, loxP sequences have not been reported to be transcriptionally active. A loss of animals due to mortality also cannot account for the unusual duration of tolerance. There was no loss of animals due to mortality in the 21-day group (26- to 28-day-old flies). However, the 28-day test (31- to 35-day-old flies) did show about 30% mortality prior to the second ethanol sedation.

Discussion

The early ethanol responses of *Drosophila* are very similar to those of humans. *Drosophila*, like humans, consume ethanol in their natural habitat and they are attracted to ethanol-containing foods (recently reviewed by Devineni & Heberlein, 2013). In addition, *Drosophila* show dopamine-dependent conditioned place-preference responses that indicate that a mildly intoxicating level of ethanol is rewarding (Kaun, Azanchi, Maung, Hirsh, & Heberlein, 2011). Furthermore, prior ethanol exposure increases the preference of flies for

ethanol-containing food (Cadieu, Cadieu, El Ghadraoui, Grimal, & Lamboeuf, 1999; Peru Y Colón de Portugal et al., 2014). In mammals, repeated ethanol exposure has been proposed to result in gradual adaptation of the nervous system that enhances the negative consequences experienced during ethanol withdrawal. Such withdrawal symptoms are thought to be able to trigger relapse (reviewed in Breese, Sinha, & Heilig, 2011). In flies, treatment with ethanol vapor that induces functional ethanol tolerance also results in an ethanol-withdrawal response (ethanol-withdrawal hyperexcitability) after ethanol clearance (Cowmeadow et al., 2006; Ghezzi, Krishnan, & Atkinson, 2014). While the adult fly acquires functional (pharmacodynamic) ethanol tolerance, it does not acquire metabolic (pharmacokinetic) ethanol tolerance. For this reason, it is ideal for studying neural adaptations that produce functional ethanol tolerance (Scholz et al., 2000).

In flies, mutant analysis has shown that a functional *slo* gene is required for the capacity to acquire functional ethanol or benzyl alcohol tolerance and that sedation with either drug induces *slo* expression. This induction appears to trigger functional tolerance that persists for about 10 days (Cowmeadow et al., 2005, 2006; Ghezzi et al., 2004). Increased expression of *slo*-encoded BK channel expression has been shown to reduce the neuronal refractory period, thereby enhancing the net excitability of CNS neurons (Ghezzi et al., 2010). While this change would be predicted to counter ethanol sedation and contribute to producing functional tolerance, it does not account for all aspects of functional tolerance, since the behavioral tolerance outlasts *slo* induction and the concomitant increase in neuronal excitability by many days. Other factors are clearly involved, and it has been suggested that the increase in BK channel activity has knock-on effects that contribute to tolerance (Ghezzi et al., 2014; Ghezzi, Pohl, Wang, & Atkinson, 2010).

We are interested in how genes sense and respond to changes in ethanol sedation to produce ethanol-induced behavior. Cells can rapidly alter histone acetylation to modulate gene activity. Increases in histone acetylation are strongly correlated with increased gene expression because this modification both makes the DNA more permissive for transcription and furthermore serves as a binding site for bromodomain-containing proteins that are part of the transcription pre-initiation complex. Many transcription factors stimulate gene expression by recruiting and/or activating histone acetyl transferases or by inhibiting histone deacetylases (Gräff & Tsai, 2013). Although quantifying mRNA abundance is a more common measure of gene activity, it is less likely to reflect changes in gene activity because of the widespread regulation of mRNA abundance by miRNAs (Béthune, Artus-Revel, & Filipowicz, 2012; Gurtan & Sharp, 2013). Because we are interested in how the genome transcriptionally responds to ethanol, we have elected to monitor histone acetylation levels and have used them as a proxy for measurements of the rate of transcription initiation. We recently genomically mapped the positions of histone H4 acetylation spikes that were common after ethanol-vapor sedation and after benzyl-alcohol sedation. The similarity between these two proved to be a very effective way to identify genes that are important for the production of functional ethanol tolerance (Ghezzi et al., 2013). However, it was not apparent that mapping histone acetylation changes in a ChIP assay would have sufficient resolution to also identify the regulatory elements themselves. Nevertheless, we observed that ethanol sedation produces a prominent acetylation spike over the 6b element.

Previously, this element had also been identified as a focus of histone acetylation following benzyl alcohol sedation. This element is conserved among *Drosophila* species and is situated between the two neural promoters that have previously been shown to be activated by sedation by either ethanol or benzyl alcohol (Bohm et al., 2000; Cowmeadow et al., 2006; Li et al., 2013). In the *slo*^{6b} stock, this 60-n DNA element has been precisely deleted from the genome by homologous recombination, and backcrossed so that it is approximately 98.5% isogenic with the parental line. Animals homozygous for the mutant allele show no effect on viability, and did not show any of the behaviors that have been previously described for mutations that interrupt the coding region of the gene or that specifically eliminate neural expression of the gene. In Li et al. (2013), removal of the 6b element was shown to greatly increase the duration of benzyl alcohol tolerance. It was observed that the mutant *slo* gene “over-reacted” to benzyl alcohol sedation, resulting in a transient induction that was greater than that produced by the wild-type gene. Although Li et al. (2013) previously showed that removal of the 6b element greatly increased the duration of benzyl alcohol tolerance, it remained to be proven that it had the same effect on ethanol tolerance.

We observed that loss of this 60-nucleotide DNA element, called 6b, significantly extends the duration of functional tolerance produced by a single ethanol sedation from ~10 days to at least 21 days. We propose that 6b is a regulatory element that has a role in the homeostatic modulation of *slo* expression in response to sedation with organic solvents. The 6b DNA element contains motifs for a number of transcription factor binding sites, but of particular interest is the heat-shock element that could be recognized by HSF transcription factors. HSF is known to interact with both HDAC1 and HDAC2 in mammals (Fritah et al., 2009; Wang et al., 2007). A hypothesis accounting for our observations here and in Li et al. (2013) is that the putative transcription factor binding sites in 6b can recruit HSF transcription factors. These factors, in turn, could recruit cofactors that depress transcription initiation from the neural promoters (perhaps HDAC1 and HDAC2 homologs), terminating expression from the neural promoters and limiting the magnitude or duration of gene induction by ethanol.

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Highlights

Histone acetylation identifies an ethanol-responsive DNA element.

Deletion of the element from the *slo* gene changes an ethanol-induced behavior.

Removal of a regulatory element increases functional behavioral tolerance to ethanol.

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Fig. 1.

Ethanol sedation induces changes in the acetylation state across the *slo* transcriptional control region. At the top of the figure is the map of the 7-kb *slo* transcriptional control region that shows the position of the evolutionarily conserved DNA regions assayed in a chromatin immunoprecipitation assay (the bulk of the coding region of the gene is not shown). The bent arrows identify transcription start sites and the adjacent open boxes are the unique 5' exons produced by each core-promoter element. The tissue specificities of promoters are identified above the arrowheads. The black box at the right is the first exon common to all *slo* transcripts. The gray boxes below the line are the evolutionarily conserved DNA elements. The two neural-specific promoters at the left are induced by sedation with organic solvents (Bohm et al., 2000; Cowmeadow et al., 2006; Ghezzi et al., 2004). The bar graphs beneath the promoter map show the changes in histone H4 acetylation state surveyed 0.5, 4, 6, 24, 48, and 96 h after ethanol sedation. Each bar corresponds to a labeled position in the promoter map. Fold change is the ratio of acetylation levels obtained

for ethanol-sedated animals and mock-sedated age-matched controls. Bar graphs represent the mean $\pm$ SEM. Statistical significance was determined by one-way ANOVA with Dunnett's comparison ($n = 3$, $*p < 0.05$, $**p < 0.01$).

The *slo*^{6b} mutant shows a substantial increase in the duration and magnitude of ethanol tolerance. **A)** Diagram depicting the paradigm for determining the duration of tolerance. The stock is said to have acquired tolerance if it recovers more rapidly from the second sedation than from the first sedation. This approach allows the recovery rates from the first and second sedations to be evaluated at the same time and minimizes differences due to day-to-day variation. After the first or mock sedation, the animals were housed in separate vials with food for 1 to 28 days and then both groups were sedated with ethanol vapor in tandem. Once sedated, the animals were switched to fresh air and the recovery rate measured. **B)** Recovery from sedation curves of populations of age-matched females after their first (light

gray) and their second (dark gray) ethanol sedation. After the flies are sedated, a fresh-air airstream flows into the vials (time = 0) and the animals are allowed to recover. Plotted is the percentage of flies that have regained postural control. For each row, the time between the first sedation and the second sedation is indicated on the far left. The response of wild-type animals is shown in the first column of plots. The response of *slo*^{6b} homozygotes is in the center column and the response of the *slo*^{55b} control animals is in the third column of plots. Each column is labeled with the genotype of the animals. Error bars are SEM, but statistical significance between the recovery curves was determined by log-rank analysis (n = 6; the numerical *p* values are provided in the plots; *indicates a *p* value of <0.05 or lower).