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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.

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, & deGraaf, 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

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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, Krishnan, & Atkinson, 2005, Cowmeadow et al., 2006). The role of BK channels in producing ethanol tolerance is not unique to flies and has also been demonstrated in mammals (Treistman & 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 nucleotide 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 $slo^{\Delta 6b}$ stock has been described in Li, Ghezzi, Pohl, Bohm, and Atkinson (2013). The construction of $slo^{\Delta 55b}$ control stock has been described in Li et al. (2015). Neither of these stocks contain any other genetic mutations. 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 1–28 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 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 L container. To determine the time course of histone 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′-ATCGAAC GAAGCGTCCAG-3′, 5′-CGACGCGCTCAAACG-3′), 4b (5′-GACCCGAT GATAAAGTCGATGT-3′, 5′-GCCAGTGACTGACTGACACACA-3′), 6b (5′-CCAGCAGCAATTGTGAGAAA-3′, 5′-CGAAGCAGACTTGAAAGCAA-3′), C1 (5′-ACAAACCAAAACGCACAATG-3′, 5′-AATGGATGAAGGACTGG GAGT-3′), cre1 (5′-GATGGGAAAGCGAAAAGACAT-3′, 5′-CATGTCCGTCAAAGCGAAAC-3′), s2 (5′-CATTGCTATCCCTTCCCATC-3′, 5′-ATGCAATGAAGCGAAGAACC-3′), 55b (5′-ACCCAATT GAATTCGCCTTGTCTT-3′, 5′-CCCACTCTCCGGCCATCTCT-3′), C2 (5′-GCACTCGACTGCACTTGAAC-3′, 5′-AATGAAAAAGTTCTCTCTGTGCAT-3′), and cre2 (5′-TGGATTGCGACCGAGTGTCT-3′, 5′-ATCAATACGA TAACTGGCGGAAACA-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 ΔΔCT method. Fold enrichment over control is equal to $2^{(Ct\ Input\ -\ Ct\ IP)}$ experimental/$2^{(Ct\ Input\ -\ Ct\ IP)}$ control. All data were normalized to Cyp1 using the primers 5′-TCTGCGTATGTGTGGCTCAT-3′ and 5′-TACAGAACTCGCGCATTCAC-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, &