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# Selective breeding for ethanol-related traits alters circadian phenotype

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ABSTRACT

Previous studies in mice and rats have shown that selective breeding for high and low ethanol preference results in divergence of circadian phenotype in the selected lines. These results indicate that some alleles influencing ethanol preference also contribute to circadian rhythm regulation. Selective breeding has also been used to produce lines of mice differing in a number of other ethanol-related traits, while studies of phenotypic and genetic correlation indicate that diverse ethanol-related traits are influenced by both shared and unshared genetics. In the present study, we examined several features of circadian activity rhythms in a mouse line selected for binge-like drinking and in mouse lines selected for high and low severity of ethanol withdrawal convulsions. Specifically, Experiment 1 compared High Drinking in the Dark (HDID-1) mice to their genetically heterogeneous progenitor line (HS/Npt), and Experiment 2 compared Withdrawal Seizure-Prone (WSP-2) and Withdrawal Seizure-Resistant (WSR-2) mice. Both line pairs displayed differences in their daily activity patterns under light–dark conditions. In addition, HDID-1 mice showed shorter free-running periods in constant light and less coherent activity rhythms across lighting conditions relative to HS/Npt controls, while WSP-2 mice showed longer free-running periods in constant darkness relative to WSR-2 mice. These results strengthen the evidence for genetic linkages between responsiveness to ethanol and circadian regulation, and extend this evidence to include ethanol-related phenotypes other than preference drinking. However, the present results also indicate that the nature of genetic correlations between and within phenotypic domains is highly complex.



## Introduction

Several lines of research have revealed bidirectional interactions between alcohol (ethanol) intake and circadian clock function at both the physiological and genetic levels. Thus, ethanol exposure alters free-running circadian period and responsiveness to phase-shifting stimuli (Mistlberger & Nadeau, 1992; Rosenwasser, Fecteau, & Logan, 2005; Rosenwasser, Logan, & Fecteau, 2005; Seggio, Fixaris, Reed, Logan, & Rosenwasser, 2009; Seggio, Logan, & Rosenwasser, 2007), in part via ethanol-induced alterations in neurotransmission (Brager, Ruby, Prosser, & Glass, 2010, 2011; McElroy, Zakaria, Glass, & Prosser, 2009; Ruby, Brager, DePaul, Prosser, & Glass, 2009, Ruby, Prosser, DePaul, Roberts, & Glass, 2009) and gene expression (Chen, Kuhn, Advis, & Sarkar, 2004; Madeira et al., 1997; Sanna et al., 1993) within the suprachiasmatic nucleus (SCN) circadian pacemaker. Conversely, both environmental perturbation of circadian rhythms (Clark, Fixaris, Belanger, & Rosenwasser, 2007; Gauvin et al., 1997; Rosenwasser, Clark, Fixaris, Belanger, & Foster, 2010) and clock gene mutations (Brager, Prosser, & Glass, 2011; Dong et al., 2011; Spanagel et al., 2005) alter voluntary ethanol intake.

Selectively bred lines of rats and mice have been used widely to reveal genetic influences on various responses to ethanol, including preference drinking, withdrawal severity, and three responses to acute ethanol—hypothermia, sedation, and locomotor stimulation (Phillips, Feller, & Crabbe, 1989). Of particular importance, selected lines can help elucidate genetic correlations among diverse ethanol-related traits. Thus, if selection for a specific phenotype also results in correlated line differences in another, non-selected trait, this suggests that the two traits share partially overlapping genetic determinants, especially if the phenotypic relationship can be replicated in multiple independently derived replicate lines (Crabbe, Phillips, Kosobud, & Belknap, 1990). For example, mice selected for high and low ethanol preference also show differential

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severity of ethanol withdrawal (low and high, respectively), while conversely, selection for high and low withdrawal results in differential ethanol preference (Metten et al., 1998). While these results are consistent with the inverse genetic correlation between ethanol preference and withdrawal seen among inbred mouse strains (Metten & Crabbe, 2005; Metten et al., 1998), the effects of selection for withdrawal severity on ethanol preference have been somewhat inconsistent across studies (Ford et al., 2011; Hitzemann et al., 2009; Kosobud, Bodor, & Crabbe, 1988).

A similar approach can also be employed to explore possible genetic correlations between ethanol-related phenotypes and neurobehavioral traits other than those directly related to ethanol responsiveness. For example, Hofstetter, Grahame, and Mayeda (2003) examined free-running circadian activity rhythms in selectively bred High Alcohol Preferring (HAP) and Low Alcohol Preferring (LAP) mice (now referred to as HAP-1 and LAP-1 respectively, due to the subsequent derivation of replicate lines), and found that HAP mice displayed shorter circadian periods in constant darkness (DD) than did LAP mice. While a more recent study failed to replicate this finding in the HAP-2 and LAP-2 lines, HAP-2 mice did display shorter free-running period during free-choice ethanol availability (Trujillo, Do, Grahame, Roberts, & Gorman, 2011). Together, these results suggest that selection for ethanol preference results in the segregation of alleles influencing a fundamental property of the underlying circadian pacemaker, its inherent period.

Rosenwasser, Fecteau, Logan, Reed, et al. (2005) examined circadian activity rhythms in two sets of selectively bred ethanol-preferring and non-preferring rat lines: the high drinking P (Preferring) and HAD-2 (High Alcohol Drinking, replicate 2) lines, and their corresponding low drinking NP (Non-Preferring) and LAD-2 (Low Alcohol Drinking) lines. While both line pairs were generated using identical selection criteria, the P/NP and HAD/LAD animals were derived from different progenitor stock and thus have dissimilar genetic backgrounds (Murphy et al., 2002). While HAD-2 rats expressed shorter free-running periods in DD than LAD-2 rats, P rats displayed shorter free-running periods than NP rats only in constant light (LL), but not in DD. Further, P rats were less able than NP rats to entrain their circadian rhythms to non-24-h light–dark (LD) cycles. Taken together, these results indicate selection for ethanol preference altered the inherent pacemaker period in HAD-2/LAD-2 rats but modified the light-responsiveness of the circadian pacemaker in P/NP rats.

In the present study, we explored possible effects of selection for ethanol-related traits other than preference drinking on circadian phenotype, including ethanol withdrawal severity and binge-like drinking to intoxication. Withdrawal Seizure-Prone (WSP-1, WSP-2) and Withdrawal Seizure-Resistant (WSR-1, WSR-2) replicate lines were selected for high and low severity of handling-induced convulsions following ethanol vapor exposure (Crabbe & Phillips, 1993; Kosobud & Crabbe, 1986). While initial reports indicated that WSP mice display reduced ethanol preference drinking relative to WSR mice (Kosobud et al., 1988), this difference seems to have largely disappeared in the current descendents of these animals, despite persistence of differential withdrawal severity (Ford et al., 2011; Rosenwasser, Fixaris, Crabbe, Brooks, & Ascheid, 2012).

Recently, replicate lines of High Drinking in the Dark (HDID-1, HDID-2) mice have been selected based on achievement of high blood ethanol concentrations (BECs) in the "Drinking in the Dark" (DID) protocol, a putative model of binge-like drinking (Crabbe, Phillips, & Belknap, 2010; Crabbe et al., 2009; Rhodes, Best, Belknap, Finn, & Crabbe, 2005). In the typical DID test, animals are offered 20% ethanol as their only fluid for a 2–4 h period during the early dark phase of the LD cycle and achieve intoxicating BECs (Crabbe et al., 2009). Across a panel of inbred strains, differences in DID correlate positively with differences in 24-h preference drinking, indicating that these two traits partially depend on shared genes (Rhodes et al., 2007). Nevertheless, despite the dramatic difference in DID drinking between HDID mice and the genetically heterogeneous HS/Npt (HS) progenitor line, these two lines display little or no difference in either 24-h preference drinking (Crabbe, Spence, Brown, & Metten, 2011; Rosenwasser et al., 2012) or acute and chronic withdrawal severity (Crabbe et al., 2012).

In the present experiments, we assayed several parameters of circadian activity rhythms under LD, DD, and LL conditions in HDID-1 and HS mice (Experiment 1) and in WSP-2 and WSR-2 mice (Experiment 2). We found that selection for both ethanol withdrawal severity and binge-like drinking results in differences in circadian phenotype, thus strengthening the evidence for genetic linkages between ethanol responsiveness and circadian regulation.

## Materials and methods

### *Subjects and apparatus*

All mice employed in these experiments were shipped to the University of Maine from breeding colonies maintained at the Portland VA Medical Center. Experiment 1 used male HDID-1 and HS mice ($N = 15$ per line) whereas Experiment 2 used male WSP-2 and WSR-2 mice ($N = 13$ per line); the two experiments were otherwise identical. HDID-1 mice were from the 18th selection generation (S18), and HS/Npt mice were from the 70th generation (G70). WSP-2 and WSR-2 mice were initially selected for 26 generations, followed by long-term unselected breeding (S26G120 and S26G121, respectively). Mice arrived in the laboratory at 6–8 weeks of age and were immediately placed individually into running-wheel cages (Coulbourn Instruments, Lafayette, IN; wheel diameter = 11.5 cm) housed within light-controlled and sound-shielded cabinets. Wheel turns were monitored continuously by microswitches mounted outside of the cage body, and running-wheel activity was recorded and analyzed using the ClockLab interface system (Coulbourn Instruments, Lafayette, IN). Food (Prolab RMH 3000) and tap water were available ad libitum.

### *Procedures*

Animals were maintained initially under a standard LD 12:12 cycle for 3 weeks, followed by an abrupt 6-h phase advance of the LD cycle, and followed 3 weeks later by an abrupt 6-h phase delay of the LD cycle. These conditions allowed us to determine the overall shape of the circadian activity waveform under stable LD conditions, and to evaluate the number of transient cycles required for animals to adapt to LD phase shifts. Next, after 3 additional weeks of LD entrainment, animals were exposed to DD for 3 weeks and finally to LL for 3 weeks. These procedures allowed us to evaluate the free-running circadian period and to determine the spectral magnitude ("coherence") of the activity rhythm in both DD and LL. Analyses of days required for phase-shifting and free-running period were performed by two independent observers in a semi-automated manner using ClockLab's onset-detection algorithm, while spectral magnitude was determined using the Lomb-Scargle periodogram. Circadian parameters were compared across lighting conditions and breeding lines using 2-factor repeated-measures ANOVA with lighting conditions as the repeated factor, and pair-wise comparisons were performed using the LSD test (SPSS, Chicago IL, USA).

### *Ethics*

These experiments were pre-approved by the University of Maine Institutional Animal Care and Use Committee (IACUC), and