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Effects of ethanol intake and ethanol withdrawal on free-running circadian activity rhythms in rats

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Abstract

Chronic alcohol intake and alcohol withdrawal are associated with dramatic disruptions of daily (circadian) biological rhythms in both human alcoholics and experimental animals. The extent to which these observations are due to pharmacological effects on the underlying circadian pacemaker is not known, however, since no human studies and very few animal studies have been conducted under free-running conditions. In the present study, free-running circadian activity (wheel-running) rhythms of rats were monitored before, during and after exposure to either 10% or 20% ethanol solution as the only drinking fluid. Across individuals, both lengthening and shortening of free-running period were observed during ethanol intake, and treatment termination led to either a return to baseline or to an exacerbation of the original ethanol effect. These variable effects appeared to be related to both ethanol concentration and to individual differences in baseline period, such that relatively short free-running period during baseline was associated with greater period-lengthening during ethanol exposure. These bidirectional affects of ethanol on free-running period are generally similar to effects seen previously with other psychoactive drugs, including antidepressants. The results of this study indicate that ethanol influences the circadian pacemaker, and that the chronobiological disruptions seen in human alcoholics may be due, in part, to alterations in circadian pacemaker regulation.

Keywords: Ethanol; Alcoholism; Circadian; Activity; Wheel-running; Rats

1. Introduction

In humans, alcohol intake and chronic alcoholism are associated with widespread and well-documented disruptions of sleep–wake cycles and other daily biological rhythms [1–7], while similar disruptions have been seen in experimental animals subjected to chronic ethanol treatment [8–12]. On the other hand, the extent to which the chronobiological effects of alcohol are mediated by pharmacological effects on the underlying circadian pacemaker is not known, primarily due to an almost-total lack of studies conducted under free-running conditions [13]. Thus, it remains possible that the disruptive effects of alcohol on circadian rhythms are due mainly to effects on neural and physiological control systems situated downstream from the pacemaker. While several alcohol-sensitive neurotransmitter and receptor systems are known to play key roles in circadian pacemaker regulation [13], the hypothesis that alcohol affects the circadian pacemaker via its action on these neural systems has been largely untested. This surprising lack of evidence stands in marked contrast to the extensive available data indicating that the central circadian pacemaker is indeed sensitive to several other classes of psychoactive drugs, including antidepressants, benzodiazepines, and psychostimulants [14].

While experimental and analytical tools have been developed to effectively "unmask" the endogenous period and phase of the human circadian pacemaker, such approaches have not yet been applied to the analysis of alcohol-induced circadian rhythm disruptions in either normal or alcoholic subjects. On the other hand, limited evidence from animal experiments suggests that chronic alcohol intake can modify a fundamental parameter of the circadian pacemaker, its free-running period. While chronic

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ethanol intake has been reported to lengthen free-running circadian period in Syrian hamsters [15–17], previous preliminary data from our laboratory suggest that free-running period may be shortened during access to ethanol drinking solutions in rats [18]. Since these apparently discrepant observations may have been due to species differences, or to any of numerous other procedural differences between the studies, the present study was designed to explore the effects of chronic ethanol ingestion on free-running circadian activity rhythms in the rat, in order to better characterize the effects of alcohol intake on the mammalian circadian pacemaker.

2. Methods

2.1. Subjects and apparatus

Male Long–Evans rats were obtained from Charles River Labs at about 60 days of age and maintained individually in running wheel cages (wheel diameter: 35 cm; Lafayette Instruments) with attached side cages. Running-wheel cages were placed within light- and sound-shielded enclosures equipped with exhaust fans and programmable lighting provided by incandescent lamps. Wheel revolutions were monitored via microswitches and a computer interface system (Dataquest III, MiniMitter), and stored in 10-min blocks for subsequent analysis. Food (Prolab RMH 3000, Lab Diets) and drinking fluid (either plain water or ethanol solution) were always freely available.

2.2. Procedures

This experiment utilized a within-subjects design, in which individual animals were studied before, during, and after ethanol treatment, to examine the effects of ethanol on free-running circadian rhythms. Animals were maintained under continuous dim red light (RR, approximately 5 lx) throughout the experiment, and exposed to one of two different sequences of ethanol treatments. Animals in squad 1 (N=8) were initially maintained on plain tap water (35 days), and then exposed sequentially to 2% (7 days), 5% (7 days) and 10% (35 days) v/v ethanol solution as the sole drinking fluid, before being returned to water (21 days). Animals in squad 2 (N=5) were maintained sequentially on plain tap water (30 days), followed by 5% (9 days), 10% (7 days) and 20% (21 days) ethanol solution, and finally returned to water (21 days). Thus, the major difference in the treatment of the two squads was that squad 2 was exposed to a higher ethanol concentration than was squad 1 (20% vs. 10%).

Data analysis: Free-running circadian periods were determined for selected three-week data samples, including (1) the last three weeks of pre-alcohol baseline (water) conditions, (2) the last three weeks of alcohol treatment (either 10% or 20% depending on squad), and (3) the first three weeks after the termination of alcohol treatment. Free-running periods were assessed using three separate widely-employed and complementary approaches: cosinor spectral analysis, chi-square periodogram analysis, and visual inspection. Cosinor spectral analysis (as implemented in the Dataquest program) estimates free-running circadian period using nonlinear least-squares regression to determine the period of the sinusoidal function that minimizes the error variance. Chi-square periodogram analysis (as implemented in the Tau software package, MiniMitter Co.) uses a nonparametric (i.e., waveform-independent) approach to determine the data-folding period that maximizes the ratio between the across-cycle variance and the within-cycle variance. In the present paper, both cosinor and periodogram analyses were used to examine test periods between 20 and 30 h in 0.10-h increments (i.e., independent spectral "intensity" estimates were obtained for periods of 20.0 h, 20.1 h, etc, up to 30.0 h). Finally, free-running periods were also estimated by the slope of straight lines visually fit to activity onsets with the assistance of an automated procedure implemented in the Tau program. Preliminary statistical analysis revealed that the three measures of free-running period were highly correlated (all pairwise $r$values>0.88), and that each measure independently revealed a very similar pattern of results. Thus, the three period estimates were averaged in order to minimize the data variance.

In addition to free-running period, the cosinor analysis was also used to derive two separate and mathematically distinct measures of the strength or robustness of circadian rhythmicity. One measure, here called "spectral magnitude", is the percentage of data variance explained by the best-fitting sinusoidal function. The second measure, referred to here as "circadian amplitude", is derived by taking the raw amplitude of the best-fitting sinusoid and dividing it by its mesor (mean level), in order to yield a measure of rhythm amplitude that is uncorrelated with overall activity levels (since activity rhythms always have minima near zero, raw cosinor amplitude is strongly correlated with activity level, but the corrected amplitude reported here is not). While spectral magnitude assays the "coherence" of the rhythm, circadian amplitude assays the peak-to-trough excursion; these two measures are generally uncorrelated with one another.

Finally, fluid intakes (water or ethanol solution) were measured on a weekly basis throughout the experiment. Since frequent weighing of the animals was impractical in this experiment due to the potential for disruption of activity rhythms, ethanol intakes in grams of ethanol per kilogram per day were estimated using body weights obtained at the conclusion of testing.

3. Results

Animals in squad 1 consumed a mean of 32.35 (SEM=1.80) ml of fluid per day during maintenance on 10% ethanol solution, and weighed 544.4 (SEM=24.0)

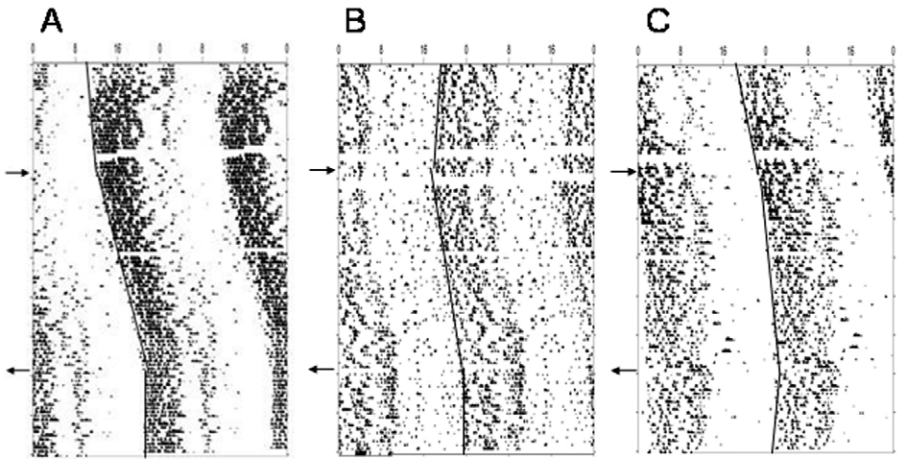

Fig. 1. Double-plotted (48-h span) activity records over the course of the experiment for three representative animals in squad 1. Animals were maintained in continuous dim red light and exposed to a 10% ethanol drinking solution, which was introduced on the day indicated by the rightward arrow and removed on the day indicated by the leftward arrow. Oblique lines superimposed on the activity records represent visual estimates of free-running period during each phase of the experiment. Both period lengthening (A,B) and period shortening (C) were observed during ethanol treatment.

grams at the end of the experiment, resulting in an estimated ethanol dose of 4.83 (SEM=0.44) g/kg/day. Animals in squad 2 consumed a mean of 22.92 (SEM=1.22) ml of fluid per day during maintenance on 20% ethanol solution, and weighed 476.6 (SEM=35.5) grams at the end of the experiment, resulting in an estimated ethanol dose of 7.70 (SEM=0.46) g/kg/day. While the difference in terminal body weights was not significant, animals in squad 2 consumed significantly less total fluid but significantly more ethanol than did squad 1 animals (independent-samples *t*-tests, both p values<0.01).

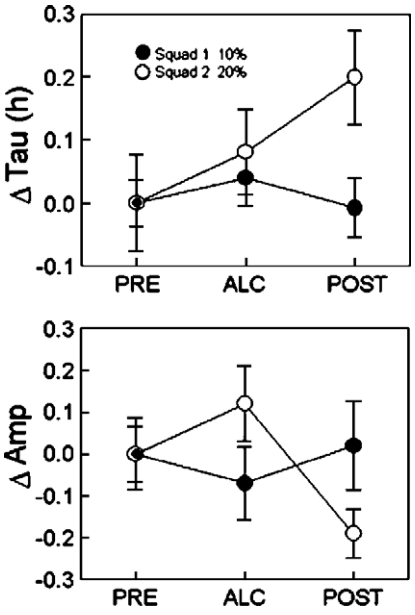

Fig. 2. Mean (±SEM) changes in free-running period (top panel) and circadian amplitude (bottom panel) as a function of ethanol treatment (ALC) and withdrawal (POST), for animals in squad 1 (exposed to 10% ethanol solution; filled circles) and squad 2 (exposed to 20% ethanol solution; open circles). The data for each squad are normalized to that squad's pretreatment value (PRE).

Inspection of activity records (Fig. 1) indicated that individual animals showed somewhat idiosyncratic responses to both the introduction and the termination of ethanol treatment. While most (8/13) animals in this experiment showed lengthening of free-running period during ethanol treatment (Fig. 1A,B), some showed no apparent change, while others showed a slight shortening of free-running period (Fig. 1C). Upon termination of ethanol treatment, most animals (Fig. 1A,B) displayed a return toward baseline, pre-treatment period, while others showed no apparent change, or even a potentiation of the original ethanol effect (Fig. 1C). In general, these changes in free-running period were apparent immediately (within a day or

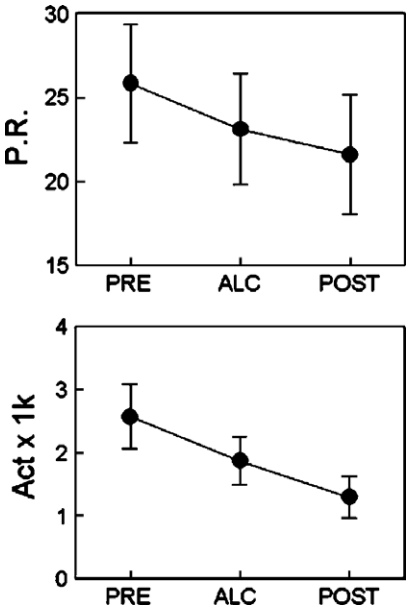

Fig. 3. Mean (±SEM) spectral magnitude (P.R., the percentage of data variance accounted for by the best-fitting cosinor function) and daily activity level (wheel-turns per day), collapsed across squads, for the three phases of the experiment.

two) following both treatment onset and treatment termination (Fig. 1A,B,C).

Two-factor repeated measures ANOVA (treatment phase: pre-ethanol, ethanol, post ethanol; squad: 1, 2) revealed significant treatment-by-squad interactions for both free-running period ($F_{2,22}$=3.22, p=0.05) and circadian amplitude ($F_{2,22}$=4.17, p=0.03), indicating that these measures were affected differentially as a function of ethanol concentration (10% vs. 20%) (Fig. 2). These findings were confirmed by separate 1-factor ANOVAs, which revealed significant (or marginal) effects of treatment phase only for squad 2 (free-running period: $F_{2,8}$=6.39, p=0.02; amplitude: $F_{2,8}$=3.90, p=0.06). Squad 1 animals showed small, non-significant lengthening of free-running period and reduced amplitude during ethanol treatment, both of which were apparently reversed upon termination of ethanol treatment. In contrast, squad 2 animals showed more substantial lengthening of free-running period during ethanol treatment, and further period lengthening after treatment termination. Additionally, squad 2 animals actually showed increased circadian amplitude during ethanol treatment, followed by a dramatic reduction in amplitude after treatment termination.

Since 2-factor ANOVA failed to indicate the presence of treatment-by-squad interactions for either spectral magnitude or daily activity level, data from the two squads were combined and subjected to 1-factor (treatment phase) ANOVA, which revealed a significant and progressive reduction in activity levels over the course of the experiment ($F_{2,24}$=6.46, p=0.006) (Fig. 3). While spectral magnitude showed similar reductions, these changes were not statistically significant.

Despite the fact that significant changes in free-running period were seen only in squad 2, additional analysis revealed that variability in period-responses to ethanol was systematically related to individual differences in baseline free-running period, across the two squads (Fig. 4). Thus, animals from both squads showing relatively short baseline periods tended to display period lengthening during ethanol treatment, while animals displaying relatively long baseline periods tended to display period shortening, during ethanol treatment.

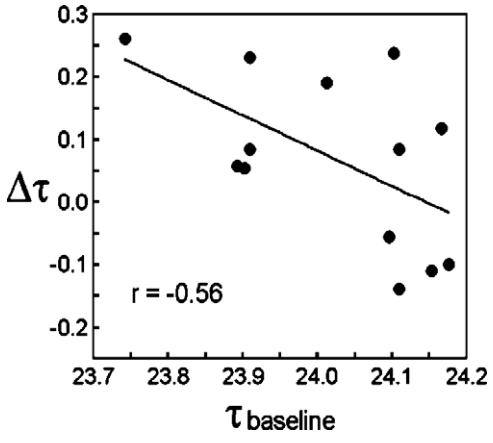

Fig. 4. Changes in free-running period ("delta–tau") during ethanol treatment (ethanol period–baseline period) as a function of baseline period, collapsed across the two squads.

4. Discussion

The results of this experiment indicate that both ethanol intake and ethanol withdrawal alter the period and amplitude of free-running circadian activity rhythms in the rat. While the effects of ethanol on circadian amplitude could potentially be mediated entirely by mechanisms downstream from the circadian pacemaker, the effects of ethanol on free-running period indicate that this drug must ultimately affect the underlying circadian pacemaker, either directly, or via action on pacemaker input (entrainment) pathways. Since previous studies have shown that the level of spontaneous running-wheel activity is generally negatively correlated with free-running period [19], one potential avenue for ethanol to indirectly affect the circadian pacemaker is via suppression of locomotor activity. This is unlikely to have been a factor in the present study, however, since both squads of animals showed similar reductions in activity across the phases of the experiment, but only the animals maintained on 20% ethanol showed progressive lengthening of free-running period.

In this experiment, both shortening and lengthening of free-running period were observed during ethanol treatment, and these effects could be either reversed or potentiated by ethanol withdrawal. Further, the effects of both ethanol intake and ethanol withdrawal on free-running period (and amplitude) appear to be dose-dependent, since their magnitude and direction differed between animals maintained on 10% and on 20% ethanol solutions. Previous reports have described ethanol-induced lengthening of free-running circadian period in Syrian hamsters [15–17], but in contrast, our laboratory had originally reported period-shortening during ethanol treatment in rats [18]. While these prior studies had many methodological differences (e.g., species, ethanol concentrations, free vs. forced intake), the present results indicate that the direction and magnitude of ethanol effects on free-running period depend on individual differences in baseline period, even for a given species and treatment protocol. Indeed, the animals in our previous study [18] showed considerably longer baseline periods than those in the present study (the mean pre-ethanol period was approximately 24.30 in the previous study and about 24.00 in the present study), which probably accounts for the consistent ethanol-induced period-shortening seen in the previous study. While these observations complicate the interpretation of ethanol's effects on the circadian pacemaker, they are actually quite similar to the reported bidirectional period-altering effects of other psychoactive agents, including antidepressants, "prodepressants", and anxiolytics [20–23].

The results of this study contribute to a body of emerging evidence linking ethanol intake to the circadian timing system. In addition to the chronobiological effects of adult ethanol treatment described in the present study, two separate groups have recently reported that perinatal ethanol exposure alters the free-running period and photic entrainment of the circadian pacemaker [24–26], and our laboratory has found that the photic responsiveness of the circadian pacemaker is also altered by adult ethanol exposure [27]. In addition, both developmental and adult ethanol exposure alter the expression of specific neuropeptides, growth factors, and circadian "clock genes", particularly within the hypothalamic suprachiasmatic nucleus (SCN), known to contain the mammalian "master" circadian pacemaker [24,28–31]. Further, the ethanol-evoked suppression of VIP (vasoactive intestinal peptide) and AVP (arginine vasopressin) expression within the SCN is exacerbated after ethanol withdrawal [28,29], providing a possible neurochemical correlate for the potentiating effects of ethanol withdrawal on free-running period seen in squad 2 animals in the present study. While the specific mechanisms underlying the pharmacological effects of ethanol on the circadian pacemaker are unknown, several ethanol-sensitive neurotransmitter and receptor systems, including both excitatory (glutamate) and inhibitory (GABA) amino acids [32], serotonin [33], and neuropeptide Y (NPY) [34], are known to be involved critically in circadian pacemaker regulation [35], suggesting that considerable work will be required to identify the mechanisms underlying these effects.

In addition to the chronobiological effects of ethanol on the circadian pacemaker, the circadian timing system also reciprocally modulates a variety of physiological responses to ethanol [36,37], as well as ethanol intake [38]. Thus, ethanol intake occurs according to a marked circadian rhythm, closely resembling the circadian profile of other ingestive and motivated behaviors [38]. Furthermore, ethanol intake and ethanol preference are modulated by photoperiod, and are increased by experimentally-induced circadian desynchrony (i.e., simulated jet lag and shiftwork schedules) [38,39]. Finally, very recent studies imply bidirectional genetic linkages between circadian regulation and ethanol intake, since selective breeding for ethanol preference alters the circadian pacemaker in both mice [40] and rats [27], while a loss-of-function mutation of the circadian clock gene, *Per 2*, increases ethanol intake and ethanol preference in mice [41].

The chronobiological effects of chronic ethanol intake in humans have been studied mainly in alcoholic patients, usually during acute withdrawal or during longer-term abstinence. These studies have revealed rather dramatic disruptions in the timing (phase), amplitude, and waveform of circadian rhythms of sleep, hormone secretion, body temperature, and other parameters, some of which may persist over considerable periods of abstinence [1–7]. Indeed, it has been suggested that persistent disruptions of sleep and circadian rhythms may promote relapse to drinking [7], possibly as a consequence of the marked anxiety and dysphoria that characterize ethanol withdrawal in both humans [42] and experimental animals [43,44]. Unfortunately, experimental protocols designed to "unmask" the phase and period of the endogenous circadian pacemaker in human subjects have not yet been applied to the study of circadian desynchrony in alcoholics. Nevertheless, the results of the present study indicate that these effects are likely to be due, at least in part, to disturbances in circadian pacemaker function.

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