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Alcohol 44 (2010) 239–244

ALCOHOL

Circadian wheel-running activity during withdrawal from chronic intermittent ethanol exposure in mice

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Abstract

Alcohol withdrawal is associated with affective–behavioral disturbances in both human alcoholics and in animal models. In general, these phenomena are potentiated by increased alcohol exposure duration and by prior withdrawal episodes. Previous studies have also reported locomotor hypoactivity during ethanol withdrawal in rats and mice, but only in novel test environments and not in the home cage. In the present study, we examined the effects of withdrawal from chronic intermittent ethanol (CIE) vapor exposure on the level and circadian periodicity of wheel-running activity in C57BL/6J mice. CIE treatment resulted in reductions in wheel-running activity compared with plain-air controls that persisted for about 1 week after withdrawal. Analysis of circadian waveforms indicated that reduced activity occurred throughout the night phase, but that daily-activity patterns were otherwise unaltered. CIE failed to alter free-running circadian period or phase in animals maintained under constant darkness. These results show that ethanol withdrawal can result in locomotor hypoactivity even in the habitual, home-cage environment, and suggest that withdrawal-related reductions in wheel-running activity may reflect the specific motivational significance of this behavior.

Keywords: Circadian; Wheel running; Ethanol; Alcohol; Withdrawal; Inbred mice; C57BL/6J

Introduction

Alcohol withdrawal is associated with severe and persistent affective–behavioral disturbances. In rodents, these disturbances are commonly modeled using measures of locomotor activity, exploration, and anxiety-like behavior. Thus, numerous studies have reported both increased anxiety and reduced locomotor activity during withdrawal from chronic ethanol exposure in rats (Overstreet et al., 2003; Rasmussen et al., 2001; Valdez et al., 2002) and mice (Hutchins et al., 1981; Kliethermes et al., 2004). Because common tests of anxiety-like behaviors, such as the elevated-plus maze and light–dark (LD) box, rely on voluntary movement and exploration within the testing apparatus, it can be challenging to isolate experimental variables reflecting anxiety from those reflecting locomotion (File, 1995). This is a potentially serious confound in studies of ethanol withdrawal, because reduced locomotor activity during withdrawal could result from nonspecific factors, such as lethargy or malaise (Kliethermes, 2005; Kliethermes et al., 2005).

On the other hand, decreased locomotor activity in a novel test environment has itself been used widely as a behavioral marker for anxiety (ie, in the open-field test; Stanford, 2007), whereas strain differences in anxiety-like behavior and locomotor activity are strongly correlated among inbred mice, suggesting that these two variables reflect partially overlapping constructs (Milner and Crabbe, 2008). Indeed, withdrawal-induced malaise is unlikely to be a major factor contributing to reduced exploratory locomotion during tests for anxiety-like behavior, because little or no locomotor suppression was seen in ethanol-withdrawn mice when assessed in their home-cage environment (Kliethermes et al., 2005). Thus, the co-occurrence of locomotor suppression and behavioral anxiety during ethanol withdrawal may reveal important biological relationships among different measures of affective behavior, rather than methodological confounding.

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0741-8329/$ – see front matter
doi: 10.1016/j.alcohol.2010.02.011

To better define the generality of ethanol withdrawal–induced hypolocomotion, we examined the effects of withdrawal from chronic intermittent ethanol (CIE) vapor on home-cage wheel-running activity. Wheel-running activity is thought to reflect a specific form of exploratory motivation, behaviorally distinct from "general activity" (Mather, 1981; Sherwin, 1998). Access to running wheels is both rewarding and reinforcing (Belke, 1997; de Visser et al., 2007; Iversen, 1993; Lett et al., 2000), can lead to compulsive, addiction-like behavior (Ferreira et al., 2006; Hoffmann et al., 1987), and has both antidepressive and anxiolytic effects in a variety of animal models (Brene et al., 2007; Duman et al., 2008; Salam et al., 2009). Furthermore, this behavior is controlled by brain mechanisms similar to those associated with the rewarding properties of drugs of abuse, including the mesolimbic dopamine–opioid system (Lett et al., 2001, 2002; Pietropaolo et al., 2008; Vargas-Perez et al., 2008; Werme et al., 2002a), and under some conditions, may modulate ethanol preference (McMillan et al., 1995; Ozburn et al., 2008; Werme et al., 2002b). We reasoned, therefore, that the assessment of wheel-running activity during ethanol withdrawal might reveal effects that were not apparent in simple measures of home-cage ambulation (Kliethermes et al., 2005).

In addition to examining the effects of ethanol withdrawal on the overall level of wheel-running activity, we also investigated possible changes in the circadian aspects of this behavior. Although human alcoholics display dramatic disruptions in sleep–wake cycles and other circadian biological rhythms during both acute withdrawal and protracted abstinence, the extent to which these effects reflect alterations in circadian pacemaker function is not known. Such a mechanism seems likely, however, because chronic ethanol intake shortens free-running circadian period (Dwyer and Rosenwasser, 1998; Rosenwasser et al., 2005a; Seggio et al., 2009) and attenuates light-induced circadian phase shifting (Rosenwasser et al., 2005b; Ruby et al., 2009; Seggio et al., 2007, 2009) in experimental animals. Nevertheless, these studies were all conducted under continuous free-choice access to ethanol, conditions that are unlikely to produce ethanol dependence. Thus, the effects of ethanol dependence and withdrawal on circadian rhythms have not been investigated in an animal model.

The current experiments were, therefore, designed to determine the effects of CIE vapor exposure on the level and circadian rhythmicity of wheel-running activity in C57BL/6J mice. We hypothesized that ethanol withdrawal would suppress locomotor activity and disrupt the expression of circadian activity rhythms, similar to the effects reported in withdrawn alcoholics.

Materials and methods

Subjects and apparatus

Upon arrival in the laboratory, the 6–8-week-old male C57BL/6J mice (Jackson Laboratories, Bar Harbor, ME) were weighed (range: 19–22 g) and placed in individual running-wheel cages (wheel diameter: 35 cm). Each running wheel cage was housed in a separate light- and sound-shielded enclosure equipped with a standard fluorescent bulb. Food (Prolab RMH 3000) and water were freely available throughout the experiment. Wheel-running activity was recorded and analyzed using the ClockLab interface system (Actimetrics Co., Wilmette, IL).

Procedures

Each animal in this experiment was exposed to both a 4-day CIE vapor–exposure protocol (see later) and a plain-air control procedure, using a repeated-measure cross-over design. All mice were initially maintained under an LD 12:12 cycle for a period of 10–14 days to allow for acclimation to running wheels and stabilization of activity levels. After this acclimation period, the animals were exposed to either CIE or air treatment. During the 4-day-exposure protocol, the running-wheel cages were moved from the normal experimental cabinet and placed within large Plexiglas inhalation chambers ($60 \times 36 \times 60$ cm). Thus, although animals had free access to their running wheels throughout the protocol, wheel-running activity was not recorded in the vapor chambers. After CIE or air treatment, running-wheel cages were returned to the normal recording cabinet, and wheel turns were recorded for an additional 3–4-week posttreatment period. During the posttreatment phase of the experiment, animals were either maintained under the original LD cycle (Groups 1 and 2) or transferred into constant darkness (DD; Group 3) to evaluate the period and phase of free-running circadian activity rhythms (see later). After 4 weeks, Group 3 (DD) animals were returned to the original LD cycle until stable re-entrainment was achieved. Finally, all animals were exposed to the second 4-d treatment protocol, either CIE or air, and again studied for a 3–4-week posttreatment period in either LD or DD. A schematic representation of the experimental timelines for the three groups is presented in Fig. 1.

Chronic intermittent ethanol protocol

The CIE protocol used in this experiment was based on the work of Becker et al. (Becker et al., 1997; Becker and Hale, 1993) and consisted of four daily cycles of 16 h of ethanol vapor exposure alternating with 8 h of plain-air exposure. Each 16-h ethanol vapor exposure started at the onset of darkness and ended 4 h into the light phase of the LD cycle. Control animals were handled identically but exposed only to plain air. Immediately before each ethanol vapor exposure, CIE animals were administered a priming injection containing 1.6 g/kg ethanol and 68.1 mg/kg pyrazole HCl, an alcohol dehydrogenase inhibitor used to rapidly increase and stabilize blood ethanol concentrations (BEC) (eg, Becker and Hale, 1993).

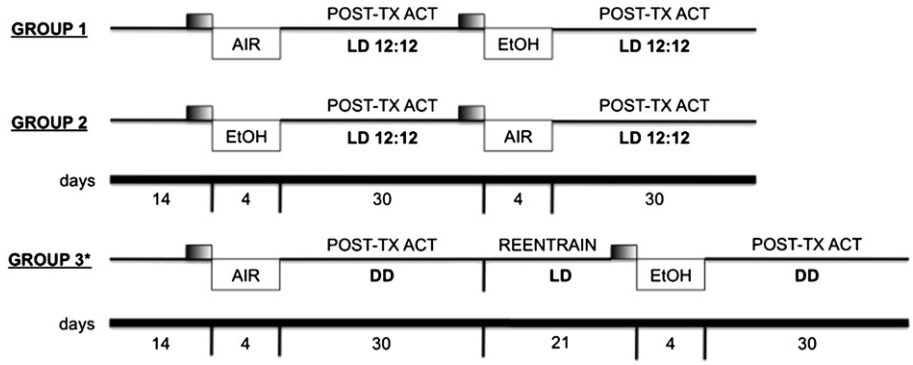

Fig. 1. Schematic illustration of repeated-measure cross-over design. Three separate groups of mice were exposed to both chronic intermittent ethanol vapor and plain air and then placed either in light–dark (LD) or dark–dark (DD) conditions to measure circadian wheel-running activity (Groups 1 and 2 were tested in LD; Group 3 was tested in DD). The duration of each experimental epoch is represented by the timeline at the bottom. Small shaded rectangle represents 5-day period of baseline activity for comparison with posttreatment activity.

Pyrazole was dissolved in 20% vol/vol ethanol solution and injected i.p. at a volume of 20 mL/kg, whereas control animals were administered an identical dose of pyrazole in 0.9% saline solution at the same volume.

Ethanol vapor and air were delivered to the exposure chambers at a rate of 10–12 L/min, ensuring adequate air flow to meet the animals' respiratory requirements. Ethanol was vaporized using a pressurized pump to push air through a porous diffusing stone submerged in a 1.0-L bottle filled with 95% ethanol.

Measurement of ethanol concentrations in tail blood and in inhalation chambers

BECs were measured in all animals at the termination of the CIE treatment protocol, immediately before being returned to the activity-recording cabinet. Briefly, each mouse was removed from its cage and placed in a plastic restraining tube, and a small (approximately 10 μL) blood sample was collected from the tip of the tail. Blood was collected directly into a heparinized capillary tube and centrifuged for 2 min to separate serum from plasma. BECs were determined from 5-μL plasma samples using an AM-1 alcohol analyzer (Analox Instr., Lunenburg, MA).

To ensure that the ethanol vapor concentrations were within an appropriate range and stable across exposure days, 5.0-mL air samples were extracted from the ethanol chambers using a 60-mL syringe mixed with 55.0-mL of room air. The diluted sample was injected into a breathalyzer (Lifeloc FC-10; Lifeloc Technologies, Inc., Wheat Ridge, CO), and the resultant readings were compared with a standardized calibration curve of known ethanol concentration to determine chamber ethanol concentration. The ethanol concentrations were maintained at 10–12 mg/L throughout the treatment protocol.

Ethical considerations

This experiment was approved by the University of Maine Institutional Animal Care and Use Committee.

Data analysis

Circadian activity rhythms

Standard raster-style circadian actograms were generated using ClockLab software for visual inspection of activity patterns (Fig. 2). Free-running periods were estimated by the slope of the straight lines fit to activity onsets with the assistance of an automated onset-detection feature of the ClockLab software. The initial phase of activity was determined by back extrapolating the fitted line to the first day after treatment. This method was used, because most, if not all, of the mice exposed to ethanol treatment displayed significantly reduced running-wheel activity on the first posttreatment day, and reliable activity onsets could not be determined.

Statistics

Wheel-running activity was compared between groups using repeated-measure analysis of variance (ANOVA) and standard *t*-tests for pairwise comparisons. The average

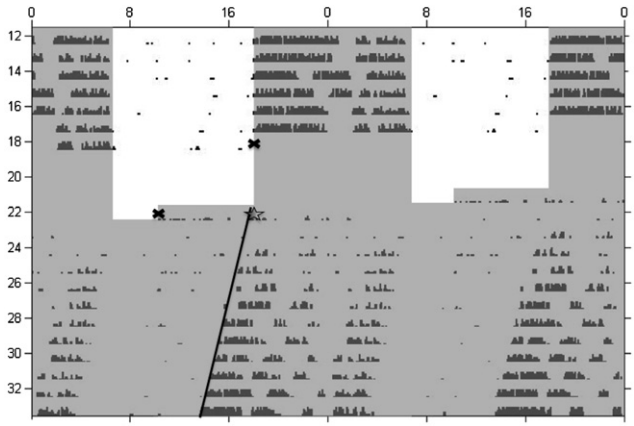

Fig. 2. Standard raster-style circadian actogram of an animal exposed to chronic intermittent ethanol vapor treatment. Lines were fitted to activity onsets on each day and back extrapolated to determine the onset on Day 1 after treatment (represented by star symbol). Shaded region denotes lights off. The x symbol represents the beginning and the end of the treatment protocol.

wheel-running activity during the final 5 days before treatment was used as the baseline, and each animal's posttreatment activity was expressed as a percentage of its own baseline to reduce the effects of individual differences in activity level. Because of the use of a crossover design, an initial analysis used both treatment (ethanol vapor vs. air) and sequence of treatment (vapor first vs. air first) as between-group factors and posttreatment day as a repeated-measure factor. Because this analysis failed to detect any significant effects or interactions related to the sequence, the results reported here are based on a subsequent analysis using only treatment and day as factors.

Results

BECs measured at the time of CIE treatment termination were within the intended target range, averaged 168.86 ± 21.86 mg/dL (mean ± standard error of the mean) across groups, and did not differ as a function of testing sequence.

Animals averaged 15,683 wheel turns per 24 h during the 5-day pretreatment baseline. For reference, this corresponds to a linear distance of 17.24 km, although the energetic demands of wheel running and locomotion on a flat surface may not be directly comparable. Collapsing across all three treatment groups, CIE treatment produced a significant reduction in wheel-running activity compared with the control air treatment that endured for about 1 week after treatment (Figs. 2 and 3). A two-factor repeated-measure ANOVA revealed a significant main effect of posttreatment day ($F_{29,754} = 6.628$, $P = .0001$) and a significant group by day interaction ($F_{29,754} = 4.36$, $P = .0001$). This interaction was explored further by conducting between-group pairwise comparisons for each posttreatment day using *t*-tests, which revealed significant group differences for the first six posttreatment days.

Averaged daily waveforms under LD 12:12 were constructed for both CIE and control animals in Groups 1 and 2 using the five pretreatment days and the first seven posttreatment days. Inspection of these waveforms showed that locomotor activity was reduced throughout the active phase of the circadian day in CIE-exposed animals but not in controls (Fig. 4). Nevertheless, there were no apparent alterations in the overall shape of the circadian activity pattern in either group.

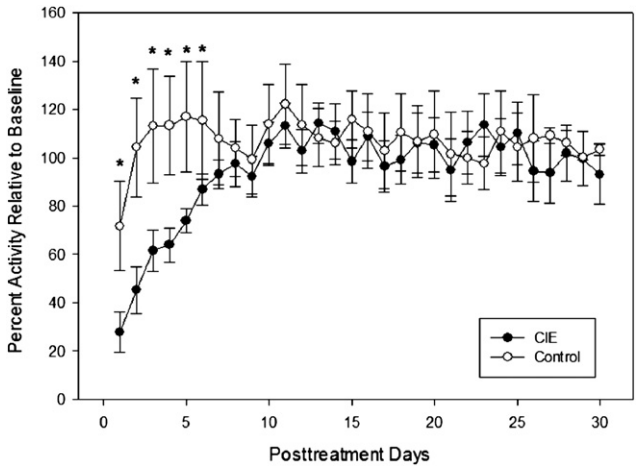

Fig. 3. Mean (±standard error of the mean) percent activity compared with that at baseline for days after chronic intermittent ethanol (CIE) vapor and plain-air treatment conditions, across all groups. A significant reduction in wheel-running activity was found for Days 1–6 in CIE-exposed mice (*$P < .05$); see text for details.

Free-running circadian period in DD and the initial phase of activity onset on posttreatment day 1 were determined for both CIE and control animals in Group 3. No significant differences between CIE and control treatments were found for either variable (Fig. 5).

Discussion

The results of this study indicate that ethanol withdrawal after CIE in C57BL/6J mice is associated with marked

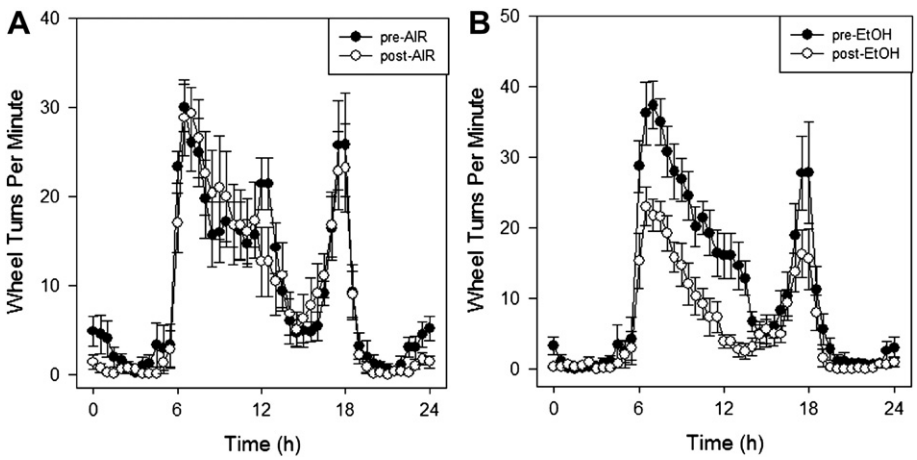

Fig. 4. Mean (±standard error of the mean) circadian waveforms for chronic intermittent ethanol (CIE) vapor and plain-air treatment conditions under light–dark setting (Groups 1 and 2). After CIE but not air treatment, reductions in wheel-running activity were exhibited in the active phase (B).

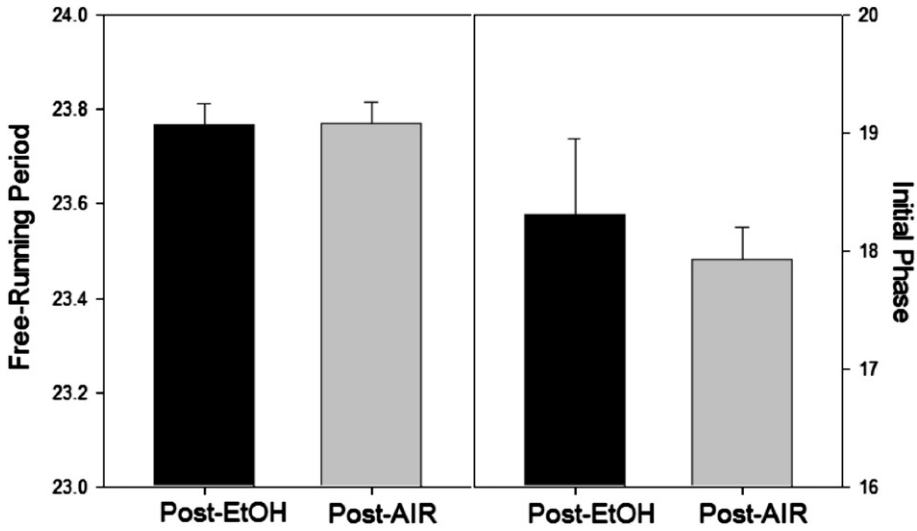

Fig. 5. Mean (±standard error of the mean) free-running period and initial phase of activity onset in dark–dark condition after chronic intermittent ethanol vapor and plain-air treatments (Group 3). No significant differences were found between ethanol- and air-exposed animals for either free-running period (left) or for initial phase of the free-running rhythm (right).

reductions in wheel-running activity that persist for about 1 week before returning to pretreatment levels. Although reduced activity was manifest throughout the circadian night phase, the overall circadian waveform was not otherwise altered. Moreover, and contrary to expectations, CIE-withdrawn mice failed to display significant alterations in free-running circadian period or phase when maintained in DD.

Although Kliethermes et al. (2004) also reported reduced locomotor activity during withdrawal from ethanol vapor exposure in mice, these observations were made during testing for anxiety-like behavior in novel test environments during the first 48 h after termination of ethanol administration. In contrast, little or no evidence for withdrawal-induced locomotor hypoactivity was seen in the home-cage environment (Kliethermes et al., 2005), suggesting that activity suppression may be specific to novel test environments. In contrast, however, ethanol-withdrawn mice in the present study showed persistent locomotor suppression in familiar, home-cage running wheels. Thus, although other procedural differences cannot be excluded (eg, ethanol vapor protocol, housing conditions, strain differences, and others), the present results indicate that the type of activity (wheel running vs. ‘‘general’’ cage activity) is a critical factor determining locomotor suppression during ethanol withdrawal.

More specifically, the pronounced reduction in wheel-running activity seen in this study may reflect the important motivational significance of this behavior, in contrast to simple home-cage ambulation. Specifically, we hypothesize that reduced wheel-running activity during ethanol withdrawal may reflect a generalized deficit in reward-seeking behavior, similar to that posited by Schulteis et al. (1995), to underlie reduced responding for brain stimulation reward in ethanol-withdrawn rats. At present, however, we cannot rule out potentially critical roles for other affective or even motoric factors, such as the amount of effort required to turn a wheel, compared with other forms of activity.

Although previous studies indicate that chronic ethanol intake alters fundamental properties of the circadian pacemaker, this was the first study to examine free-running circadian activity rhythms during ethanol withdrawal, using an experimental protocol shown to produce ethanol dependence. In contrast to our previous results indicating that free-running circadian period is shortened during chronic forced intake of 10% ethanol solution in C57BL/6J mice (Seggio et al., in press), the present study failed to detect any effect on circadian period or phase after withdrawal from CIE. Because more extended CIE exposures have been shown to lead to more dramatic and enduring effects in other withdrawal-related phenotypes (Becker et al., 1997; Becker and Lopez, 2004; Lopez and Becker, 2005), we are presently conducting additional experiments to determine if CIE effects on the circadian clock may also be revealed by such procedural modifications. In addition, because the C57BL/6J strain used in the present experiment is known for its relative insensitivity to ethanol withdrawal in other phenotypic domains (Metten and Crabbe, 2005; Metten et al., 1998), it is possible that more substantial effects on both the level and the circadian periodicity of wheel-running activity might be revealed in more withdrawal-sensitive strains.

In conclusion, we have shown that ethanol withdrawal after a 4-day CIE protocol leads to a persisting reduction in the level of home-cage wheel-running activity but fails to alter the circadian periodicity of this behavior in C57BL/6J mice. Subsequent experiments will further explore relationships between these variables and other withdrawal-related phenotypes, and their possible dependence on genetic background.

Acknowledgments

This work was supported by Integrative Neuroscience Initiative on Alcoholism-Stress (INIA-Stress), funded by the cited award U01 AA13641, PI: K. Grant, Oregon Health and Science University (OHSU).

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