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CHRONIC ETHANOL INTAKE ALTERS CIRCADIAN PERIOD-RESPONSES TO BRIEF LIGHT PULSES IN RATS

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Although chronic alcohol intake is associated with widespread disruptions of sleep-wake cycles and other daily biological rhythms in both human alcoholics and experimental animals, the extent to which the chronobiological effects of alcohol are mediated by effects on the underlying circadian pacemaker remains unknown. Nevertheless, recent studies indicate that both adult and perinatal ethanol treatments may alter the free-running period and photic responsiveness of the circadian pacemaker. The present experiment was designed to further characterize the effects of chronic ethanol intake on the response of the rat circadian pacemaker to brief light pulses. Ethanol-treated and control animals were exposed to 15-min light pulses during either early or late subjective night on the first day of constant darkness following entrainment to a 12:12 light-dark cycle. Relative to pulses delivered during early subjective night and to "no-pulse" conditions, light pulses delivered during late subjective night resulted in period-shortening after-effects under constant darkness, but only in control animals, not in ethanol-treated animals. These results indicate that chronic ethanol intake reduces the responsiveness of the circadian pacemaker to acute photic stimulation, and suggest that the chronobiological disruptions seen in human alcoholics are due in part to alterations in circadian pacemaker function.

Keywords Circadian pacemaker, Alcohol, Light pulses, Free-running period, Rodent models

INTRODUCTION

Chronic alcohol intake is associated with widespread disruptions of sleep-wake cycles and of other daily biological rhythms in both human alcoholics (Sano et al., 1993; Fonzi et al., 1994; Schmidz et al., 1996; Mukai et al., 1998; Brower, 2001) and experimental animals (Rouhani et al., 1990; Rajakrishnan et al., 1999; Ehlers and Slawecki, 2000; El-Mas and Abdel-Rahman, 2000; Danel and Touitou, 2004). Despite these well-documented disruptions, the extent to which the chronobiological effects of alcohol are mediated by effects on the underlying circadian pacemaker

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is not known, primarily as a consequence of the historical lack of studies conducted under free-running conditions (Rosenwasser, 2001). Thus, although several ethanol-sensitive neurotransmitter and receptor systems are known to play key roles in circadian pacemaker regulation (Rosenwasser, 2001), the hypothesis that alcohol intake affects the circadian pacemaker via its action on these neural systems remains 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 mood-altering psychoactive drugs, including antidepressants (Wollnik, 1992; Klemfuss and Kripke, 1994; Duncan et al., 1998), benzodiazepines (Turek and Losee-Olson, 1986; Subramanian and Subbaraj, 1996), and putative depressogenic agents (Rosenwasser, 1996).

Despite the development of analytical tools that effectively "unmask" the endogenous period and phase of the human circadian pacemaker (Czeisler, 1995), 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, emerging evidence from animal experiments suggests that both adult (Mistlberger and Nadeau, 1992; Dwyer and Rosenwasser, 1998; Rosenwasser et al., 2005) and perinatal (Earnest et al., 2001) ethanol treatment can modify the free-running circadian period, a fundamental parameter of the circadian pacemaker. Indeed, chronic ethanol treatment can evoke both lengthening and shortening of free-running circadian period (Rosenwasser et al., 2005), similar to the period-altering effects of chronic treatment with other mood-altering drugs (Rosenwasser, 1996; Subramanian and Subbaraj, 1996; Rosenwasser and Wirz-Justice, 1997).

In addition to their effects on free-running period, these same drug treatments also modify the response of the circadian pacemaker to light, including the acute phase-shifting effects of brief light pulses (Subramanian and Subbaraj, 1996; Duncan et al., 1998; Dwyer and Rosenwasser, 2000), the period-lengthening effect of constant light (Dwyer and Rosenwasser, 2000), and the phase of steady-state entrainment under daily light-dark cycles (Tamarkin et al., 1983). Thus, our study was designed to more completely characterize the chronobiological effects of ethanol by examining the effects of chronic ethanol treatment on the response of the circadian pacemaker to brief light pulses in rats.

METHODS

Subjects and Apparatus

Male Long-Evans rats were obtained from Charles River Laboratories (Wilmington, Massachusetts) and maintained individually in running-

wheel cages (wheel diameter: 35 cm; Lafayette Instruments, Lafayette, Indiana) 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 Co., Bend, Oregon), and stored in 10-min blocks for subsequent analysis. Food and drinking fluid (either plain water or ethanol solution) were always freely available. The experiments were conducted following the guidelines of the Journal for the ethical study of biological rhythm phenomena of animal models (Touitou et al., 2004).

Procedures

This experiment utilized a between-groups design to compare the effects of brief light pulses on free-running rhythms in separate groups of ethanol-treated and control (water) animals ($n = 6$ per group). Animals in both groups were initially maintained under a light-dark cycle (LD 12:12), during which ethanol drinking solution was introduced gradually to the experimental group (5% for 7 days, 10% for 8 days, and 20% thereafter). Weekly fluid intakes were recorded for animals in both groups throughout the course of the experiment. After introduction of the 20% ethanol solution to the experimental animals, animals in both groups were subsequently maintained under alternating epochs of LD 12:12 and constant darkness (DD) (LD1, 23 days; DD1, 12 days; LD2, 16 days; DD2, 15 days; LD3, 16 days; DD3, 15 days; LD4, 21 days, DD4, 15 days). Brief light pulses (15 min, approximately 50 lux) were administered on the first complete day of DD1, DD2, and DD3; as a control procedure, no light pulses were administered at the beginning of DD4. Pulses were delivered at either Zeitgeber Time (ZT) 14 (DD1) or ZT 21 (DD2 and DD3). By convention, ZT 14 refers to a time 2 h after the *projected* time of lights-off, while ZT 21 refers to 9 h after projected lights-off. Thus, in this experiment, light pulses were delivered after either 26 (ZT 14) or 35 (ZT 21) h of darkness. This procedure is a form of the so-called "Aschoff Type-2" protocol, designed to ensure that animals are stably entrained to the LD cycle at the beginning of each DD test phase, and commonly used for assessment of both photic and nonphotic phase-shifting stimuli. Although this experiment was originally designed to assess the acute *phase-shifting* effects of the light pulses, difficulties in reliably determining the phase of rat activity onsets across days, coupled with the use of relatively brief DD exposures, made this impractical. Instead, we assessed the effects of the light pulses on the *free-running circadian period* as expressed during the subsequent DD epoch, as described below.

Data Analysis

The free-running circadian period was determined for each DD epoch using three widely employed and complementary approaches: cosinor spectral analysis, χ^2 periodogram analysis, and visual inspection. Cosinor analysis was performed using the routine included with the Dataquest program, while periodogram analysis was performed using the routine included in the *Tau* software package (MiniMitter, Bend, Oregon). Visual-graphical estimates of free-running period were obtained with the assistance of an automated procedure implemented in the *Tau* program. The period estimates obtained by the three procedures were generally in good agreement, and in fact, preliminary statistical analyses produced similar overall findings for each of the three measures considered separately. Thus, to minimize the variance of period estimates, the three measures were averaged to derive the period values reported in this paper. These values were subjected to a 2×4 two-factor repeated-measures ANOVA (group: ethanol-treated, control; DD test trial: DD1, DD2, DD3, DD4), followed by post-hoc tests for simple main effects as appropriate.

RESULTS

Figure 1 presents selected excerpts from the locomotor activity records of one control and one ethanol-treated animal, illustrating typical responses to light pulses presented at ZT 14 (LD1, DD1) and ZT 21 (LD2, DD2). Representative of all animals in this experiment, these records demonstrate relatively stable steady-state entrainment under LD and free-running circadian rhythmicity under DD. While both animals showed free-running periods in DD very close to 24 h following the ZT 14 light pulse, light pulses delivered at ZT 21 generally resulted in shorter, <24 h periods under DD. Further, free-running periods following ZT 21 light pulses were typically shorter in control than in ethanol-treated animals, in both DD2 and DD3.

Mean ($+$SEM) free-running periods for both groups across the four different DD test trials are presented in Figure 2. As suggested by inspection of the activity records (Figure 1), ANOVA revealed a significant main effect of test trial ($p = 0.0001$), indicating that free-running periods differed across the different light-pulse testing conditions, and a significant group-by-test trial interaction ($p = 0.004$), indicating that ethanol-treated and control animals differed in their response to the different light pulses. As a follow-up analysis, separate one-factor (test trial) ANOVAs were computed for each group, revealing a significant effect of test trial on the free-running period in control ($p = 0.0001$) but not in ethanol-treated animals. Finally, separate pairwise comparison of the two groups

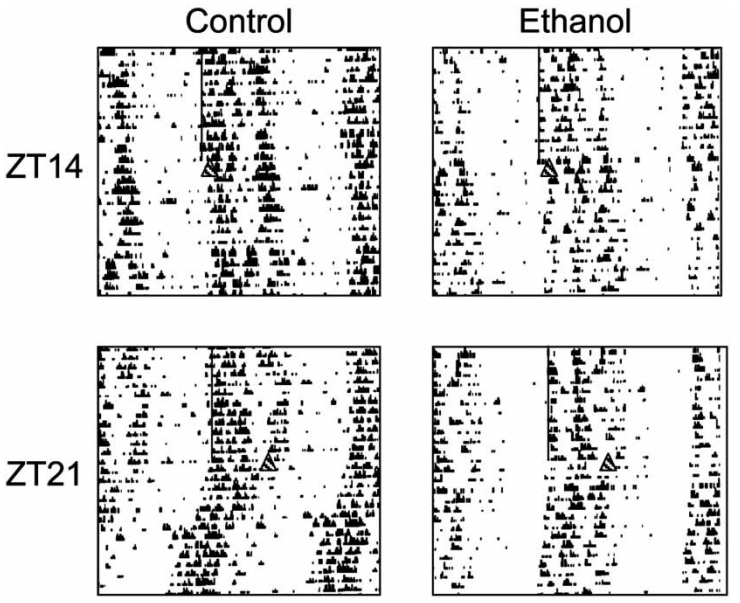

FIGURE 1 Segments of double-plotted (48 h span) activity records from one control (left) and one ethanol-treated (right) animal, illustrating typical responses to light pulses delivered at either Zeitgeber Time (ZT) 14 (top) or ZT 21 (bottom), on the first complete day of constant DD following entrainment to a 12 : 12 LD cycle. Superimposed vertical lines show the onset of darkness under the LD cycle, and the triangular symbols show the times of the light pulses. Both animals displayed similar free-running periods in constant DD following the ZT 14 light pulse, but the control animals displayed a marked period-shortening aftereffect relative to the ethanol-treated animal following the ZT 21 light pulse.

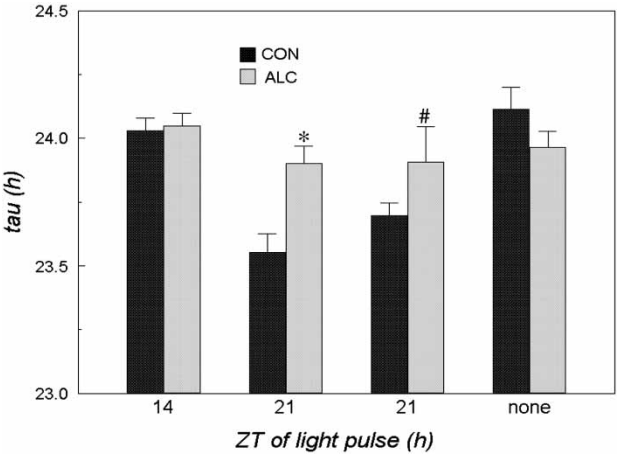

FIGURE 2 Mean (+ SEM) free-running period under constant darkness (DD) for the Zeitgeber Time (ZT) 14 (DD1), both ZT 21 (DD2 and DD3), and no-pulse (DD4) conditions. Control animals (CON; black bars) displayed significant shortening of free-running period following ZT 21 light pulses, but ethanol-treated animals (ALC; gray bars) did not. *, $p = 0.005$; #, $p = 0.07$. See text for additional statistical analyses.

for each test trial showed a significant group difference for trial 2 ($p = 0.005$) and a near-significant difference for trial 3 ($p = 0.07$). To summarize, light pulses presented at ZT 21 resulted in shortening of the free-running circadian period in DD relative to either ZT 14 light pulses or to no-pulse conditions, but only for control animals.

Ethanol-treated animals consumed significantly less fluid than did control animals over the course of exposure to 20% ethanol drinking solution (controls: 46.82 mL/day; ethanol-treated: 24.84 mL/day, $p = 0.001$). While we cannot rule out a possible contribution of dehydration to the effects described here, there were no apparent differences in general body condition of the two groups at the end of the experiment, and we previously found no differences in body weight growth curves between similarly treated ethanol-maintained and control rats housed under standard colony conditions (unpublished observations). Ethanol-treated animals in this experiment ingested an average of 3.93 g/day ethanol, and based on body weight curves measured in our colony-maintained animals, we can estimate that the treated animals in the present study consumed about 8 g/kg/day ethanol.

Previous research (Hiller-Sturmhofel and Kulkosky, 2001; Rosenwasser et al. unpublished observations) indicates that under conditions of 24 h access, rats consume ethanol solutions in a series of small bouts, closely resembling the normal nocturnal pattern of water intake. Based on published work, the level of ethanol intake achieved in this study would be expected to produce maximal blood ethanol levels of about 50 to 100 mg/dL during early subjective night, with near-zero levels during most of the circadian daytime (Aalto, 1986; Murphy et al., 2002). At no time did we observe any gross differences in the behavior of ethanol-treated and control animals over the course of this study.

DISCUSSION

The results of this experiment indicate that chronic ethanol intake alters the response of the rat circadian pacemaker to brief light pulses. While the effects of brief light pulses on the circadian pacemaker have usually been evaluated with respect to their ability to evoke acute circadian phase shifting, this study focused instead on the longer-lived "after-effects" of brief light pulses on the free-running circadian period. In fact, it has long been recognized that acute perturbation of the circadian pacemaker can induce long-lasting period aftereffects, such that light pulses delivered during early subjective night result in both an acute phase-delay as well as long-term period-lengthening, while light pulses delivered during late subjective night result in an acute phase-advance accompanied by long-term period-shortening (Pittendrigh and Daan, 1976). Indeed, recent analyses have emphasized the potential importance

of such period-responses in ensuring stable entrainment under natural conditions (Beersma et al., 1999; Daan, 2000; Daan and Aschoff, 2001; Sharma and Daan, 2002). In the present study, we analyzed period-responses to light pulses rather than phase-responses because they proved to be more reliably quantifiable under our experimental conditions, and as expected, we found significant period-shortening after late-night (ZT 21) light pulses in control animals. In contrast, since control animals did not display differences in free-running period following early-night (ZT 14) light pulses relative to no-pulse conditions, we conclude that the early-night pulses employed in this study did not evoke period aftereffects. Thus, while the present results indicate that chronic ethanol treatment blocks period after-effects resulting from late-night light pulses, they are inconclusive with regard to whether such an effect would also be seen for effective early-night light pulses.

In general, the effects of chronic ethanol treatment on free-running circadian rhythms appear quite similar to those of other mood-altering drugs. For example, alterations in free-running circadian period have been described during chronic administration of several different classes of antidepressants (Wollnik, 1992; Klemfuss and Kripke, 1994; Rosenwasser and Wirz-Justice, 1997; Duncan et al., 1998), the anxiolytic benzodiazepine, diazepam (Subramanian and Subbaraj, 1996), the putative depressogenic agent, clonidine (Rosenwasser, 1996), and ethanol (Dwyer and Rosenwasser, 1998; Rosenwasser et al., 2005). Surprisingly, both lengthening and shortening of free-running period have been observed, even for a given agent, and these effects appear to be modulated by a variety of factors, including both lighting conditions and individual differences in baseline period (Rosenwasser, 1996; Rosenwasser et al., 2005). In contrast, the reported effects of mood-altering drugs on the response of the circadian pacemaker to light pulses have been more consistent: despite their diverse pharmacological effects, clorgyline, lithium (Duncan et al., 1998), diazepam (Subramanian and Subbaraj, 1996), clonidine (Dwyer and Rosenwasser, 2000), and ethanol (present data) all appear to blunt the pacemaker's responsiveness to light. In addition, two laboratories have recently reported blunting of the circadian pacemaker's response to photic stimuli in adulthood following perinatal ethanol treatments (Sei et al., 2003; Farnell et al., 2004).

Taken together with our previous findings that chronic ethanol intake alters circadian period during long-term maintenance in free-running conditions (Dwyer and Rosenwasser, 1998; Rosenwasser et al., 2005), the present observations indicate that the chronobiological effects of ethanol are due, at least in part, to alterations at the level of the underlying circadian pacemaker. Unlike effects of ethanol on circadian phase, amplitude and waveform under steady-state entrainment that have been amply documented in both human alcoholics and experimental animals, it is

widely accepted that the effects of a drug (or other stimulus) on the expressed free-running circadian period must reflect changes in the period of the circadian pacemaker, either as a result of direct pharmacological interaction with pacemaker neurons, or via action on one or more of the pacemaker's input (entrainment) pathways. Thus, while the present results demonstrate that chronic ethanol intake alters circadian pacemaker function, they do not prove that ethanol exerts these effects via direct interaction with the hypothalamic suprachiasmatic nucleus (SCN), known to be the site of the primary mammalian pacemaker (Rosenwasser, 2003).

While the specific sites and mechanisms by which ethanol influences the circadian pacemaker are unknown, it is clear that several ethanol-sensitive neurotransmitter systems participate in circadian pacemaker regulation. Thus, chronic ethanol treatment reduces the expression of several neuropeptides within the SCN (Madeira et al., 1997). In addition, serotonin interacts bidirectionally with ethanol intake (LeMarquand et al., 1994), and a robust midbrain serotonergic projection to the SCN modulates both the free-running period and light response of the SCN circadian pacemaker (Rosenwasser, 2003). Similarly, the neurobehavioral effects of ethanol are mediated partially via indirect agonist effects on the gamma-aminobutyric acid (GABA)-A receptor (Faingold et al., 1998), and GABA-A receptors on SCN pacemaker neurons modulate both circadian period and photic responsiveness (Rosenwasser, 2003). Finally, ethanol also evokes indirect antagonist effects at the NMDA-type glutamate receptor (Faingold et al., 1998), which plays a critical role in transducing photic signals arising from the direct retinal-hypothalamic tract innervating the SCN (Rosenwasser, 2003). Further identification of the mechanisms by which ethanol influences the circadian pacemaker could eventually prove useful in the management of sleep and other chronobiological disruptions seen commonly in human alcoholics.

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