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Circadian and Genetic Influences on Tissue Sensitivity and Sleep Time to Ethanol in LS and SS Mice

DAVID M. GILLIAM[2] AND ALLAN C. COLLINS[1,3]

Alcohol Research Center and Institute for Behavioral Genetics, University of Colorado, Boulder, CO 80309

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Gilliam, D. M. and A. C. Collins. *Circadian and genetic influences on tissue sensitivity and sleep time to ethanol in LS and SS mice.* PHARMACOL BIOCHEM BEHAV 18(5) 803–808, 1983.—Circadian variations in response to ethanol were studied in long-sleep (LS) and short-sleep (SS) mice. Each LS animal received a 2.5 g/kg intraperitoneal ethanol injection, while the SS animals were injected with 4.1 or 5.0 g/kg. Different groups of mice were assessed for sleep time, waking blood alcohol concentration (BAC), and waking brain ethanol concentration (BREC) at 03.00, 09.00, 15.00, or 21.00 hr. Sleep times, waking BACs, and waking BRECs showed circadian variations in the LS mice. SS animals given the 4.1 g/kg dose showed circadian variations for waking BAC and waking BREC, but not for sleep time. The observed variations in the physiological parameters for these animals may have been confounded by a short sleep time so that they reflected circadian variations in drug absorption and/or distribution rather than in CNS sensitivity. SS mice given the 5.0 g/kg dose slept longer than those given the 4.1 g/kg dose and did not show circadian variations for sleep time, waking BAC, or waking BREC. These results suggest both circadian and genetic influences on tissue sensitivity to ethanol.

Circadian Ethanol Sleep time Brain ethanol concentrations Blood alcohol concentration Selected mice

CIRCADIAN variations in behavioral responses to pharmacological agents are well known [25,27]. For example, several studies [14, 17, 19, 21] have found that the behavioral effects of anesthetics exhibit circadian rhythms. The rhythmic variation of response to anesthetic agents has been attributed to variations in drug disposition (absorption, metabolism, and excretion) [18,24] or in tissue sensitivity [26,27].

The physiological effects of ethanol also appear to be influenced by circadian rhythms. Circadian influences on ethanol's effects have been reported in humans [9] and in mice [7, 8, 11, 13]. Human research has suggested that circadian variations in response are due, at least in part, to differences in rate of ethanol elimination [10, 30, 32, 35], and studies with rats have led to similar conclusions [23, 28, 31, 33]. In mice, however, even though circadian influences on ethanol-induced alterations in locomotor activity [2,13] and hypothermia [2, 7, 11] have been detected, those investigators who have measured ethanol metabolism suggest that their data do not indicate a role for altered ethanol metabolism in the circadian influence on ethanol response [2,11].

The present study was designed to investigate circadian variations in ethanol-induced sleep time of LS and SS mice, which have been selectively bred for differences in duration of ethanol-induced narcosis [1,15]. Because of the suggestion of circadian variations in tissue sensitivity to anesthetic agents [19,26], we also wished to obtain measures of waking blood alcohol concentration (BAC) and waking brain ethanol concentration (BREC) at different times of the day. The variations observed in sleep time, BAC, and BREC are discussed in terms of possible circadian influences on the pharmacokinetics of ethanol and on tissue sensitivity.

METHOD

Male LS and SS mice obtained from our breeding laboratory were used throughout the study. The animals in Experiment 1 were from the 20th and 21st selected generations; those used in Experiment 2 were from the 24th selected generation. Subjects were tested at 70±10 days of age, and littermates were distributed among all time points. The normal 12-hr light cycle (07.00–19.00 hr) was maintained throughout the experiments. Ambient temperature was 22±5°C.

INJECTION PROCEDURE

Experiment 1

Different groups of LS and SS mice were injected intraperitoneally (IP) with 2.5 or 4.1 g/kg ethanol, respectively, at 03.00, 09.00, 15.00, or 21.00 hr. Five to seven mice from each line were tested together at each time point on different days.

[1]Requests for reprints should be addressed to Dr. A. C. Collins, Institute for Behavioral Genetics, University of Colorado, Boulder, CO 80309.

[2]Department of Psychology.

[3]School of Pharmacy.

The 2.5 g/kg dose was administered in an injection volume of 0.01 ml/g body weight; the 4.1 g/kg dose was administered in an injection volume of 0.013 ml/g body weight. These doses of ethanol were used in the hope that the lines would exhibit approximately equal sleep times. Animals were measured for sleep time (ethanol-induced narcosis) and for waking blood and brain ethanol concentrations as described below.

Experiment 2

Due to the short sleep times observed for the SS mice in Experiment 1, and the possibility that some of the observed variations in sleep time, waking BAC, and waking BREC may have been due to variations in absorption and/or distribution throughout the day, we designed a second experiment to examine the circadian variations of these parameters in SS mice following a larger dose of ethanol. The larger dose was intended to achieve a longer sleep time and thus circumvent problems arising from animals awakening on the ascending limbs of the BAC and BREC curves. Different groups of SS mice were injected IP with 5.0 g/kg ethanol at 03.00, 09.00, 15.00, or 21.00 hr. This dose of ethanol was administered in an injection volume of 0.02 ml/g body weight. Thus, the 25.0% w/v ethanol concentration used for the SS mice in Experiment 2 was equal to that used for the LS mice in Experiment 1. Sleep time and waking BAC and BREC were measured.

SLEEP TIME

Following ethanol injection, mice were judged to have lost the righting reflex when they could not right themselves three times within a 60-sec period after being placed on their backs in U-shaped plastic troughs. Mice were judged to have regained the righting reflex when they could right themselves three times within a 60-sec period. Elapsed time between loss and recovery of the righting reflex was recorded as sleep time.

BLOOD AND BRAIN ETHANOL DETERMINATION

Two, 10-μl blood samples were obtained from each mouse by puncture of the retro-orbital sinus with a capillary pipette (Volupette) immediately after the mouse regained the righting reflex. Blood samples were mixed with a 990 μl standard isopropyl alcohol-water mixture and kept on ice until assayed for ethanol content using head-space gas chromatography [22]. Drawing of the blood samples was followed by cervical dislocation, whereupon the brain was immediately removed and divided longitudinally. Replicate brain samples were thus obtained from each mouse. Brain halves were weighed, homogenized in 4 volumes of 0.1 N perchloric acid, and centrifuged at 10,000 × g for 10 min. Fifty-microliter aliquots of the supernatant obtained from each brain were added to 950 μl of an isopropyl alcohol-water mixture. These samples were maintained on ice until assayed for ethanol by head-space gas chromatography [22]. Mean blood and brain ethanol concentrations were calculated for each mouse from the replicate samples.

ANALYSES

Data on the three variables (sleep time, waking BAC, and waking BREC) were analyzed separately across time within each line using one-way analyses of variance followed by the Student-Newman-Keuls test for comparison between means. Sleep time, waking BAC, and waking BREC were then subjected to multivariate analysis of variance using the Wilks test of significance [12]. Pearson product moment correlation coefficients were also determined for sleep time, waking BAC, and waking BREC at each time point. These analyses were performed using the Statistical Package for the Social Sciences (SPSS) [20].

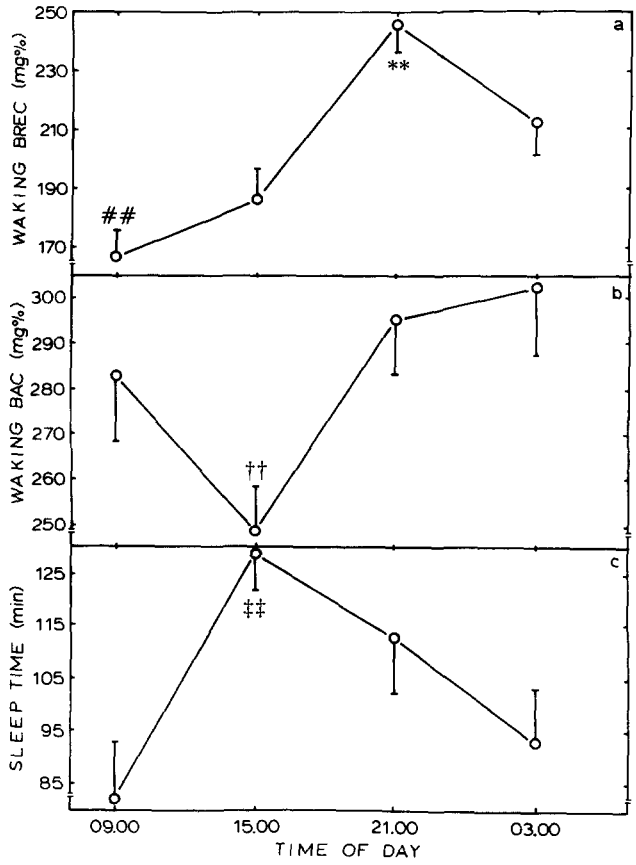

FIG. 1. Mean (a) waking brain ethanol concentrations (mg%=mg ethanol/100 g brain), (b) waking blood alcohol concentrations (mg%=mg ethanol/100 ml blood), and (c) sleep times for LS male mice injected with 2.5 g/kg ethanol at 09.00, 15.00, 21.00, or 03.00 hr. N=10 animals per group. Bars represent standard errors; $^{**}p<0.01$ when compared with BREC at 09.00, 15.00, and 03.00 hr; $\#\#p<0.01$ when compared with BREC at 21.00 and 03.00 hr; $\dagger\dagger p<0.01$ when compared with BAC at 21.00 and 03.00 hr; $\ddagger\ddagger p<0.01$ when compared with sleep time at 09.00 and 03.00 hr.

EXPERIMENT 1

RESULTS

LS Mice

Figure 1 shows mean LS sleep times, waking BACs, and waking BRECs as a function of time of day. All three variables were found to differ significantly across time: sleep time, $F(3,36)=4.4$, $p<0.01$; waking BAC, $F(3,36)=3.3$, $p<0.05$; waking BREC, $F(3,36)=11.8$, $p<0.001$. Multivariate analysis of variance revealed a significant variation over time for these variables taken collectively, $F(9,83)=6.5$, $p<0.001$, and "brain ethanol concentration" was found to be the most

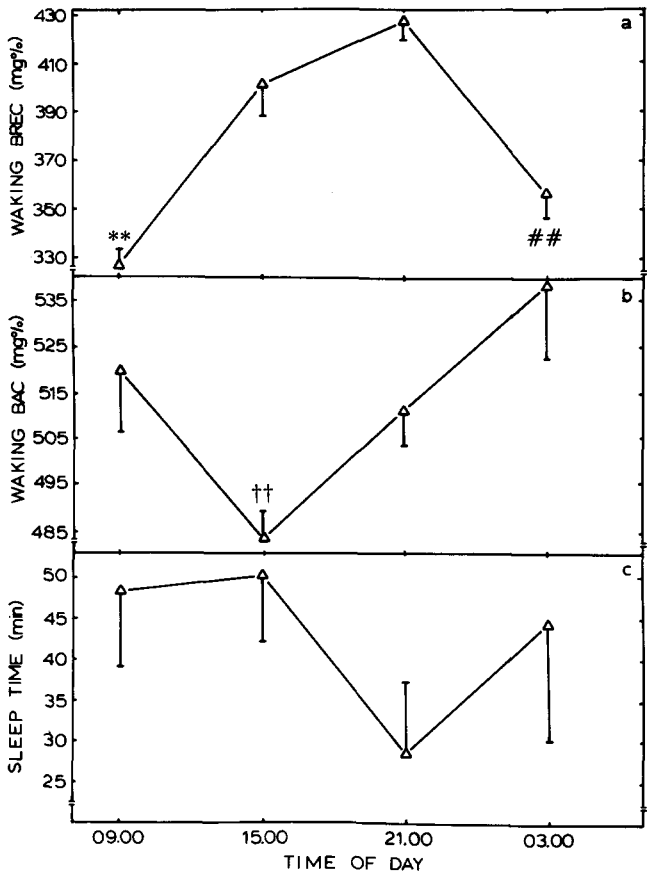

FIG. 2. Mean (a) waking brain ethanol concentrations (mg%=mg ethanol/100 g brain), (b) waking blood alcohol concentrations (mg%=mg ethanol/100 ml blood), and (c) sleep times for SS male mice injected with 4.1 g/kg ethanol at 09.00, 15.00, 21.00, or 03.00 hr. N=9 to 11 animals per group. Bars represent standard errors; $^{**}p<0.01$ when compared with BREC at 15.00, 21.00, and 03.00 hr; $\#\#p<0.01$ when compared with BREC at 15.00 and 21.00 hr; $\dagger\dagger p<0.01$ when compared with BAC at 03.00 hr.

important variable in discriminating among time points; first discriminant function coefficient=+1.22 for BREC, +0.62 for sleep time, and −0.39 for BAC.

Pearson product moment correlation coefficients were determined for sleep time and waking blood and brain ethanol concentrations at each time point. Significant correlations were found between sleep times and waking BACs at 09.00, 15.00, and 21.00 hr ($r=-.79$, $-.77$, and $-.76$, respectively; $p<0.01$), indicating that the longer sleeping mice awakened with lower BACs. Sleep times also showed significant correlations with waking BRECs at 15.00 and 21.00 hr ($r=-.79$ and $-.77$, respectively; $p<0.01$). Waking blood and brain ethanol concentrations were significantly correlated at all time points: 03.00, $r=+.61$; 09.00, $r=+.73$; 15.00, $r=+.73$; 21.00, $r=+.65$; $p<0.05$). This result reflects the fact that LS mice are well into the elimination phase of ethanol metabolism when they regain the righting reflex; blood and brain ethanol concentrations are therefore decreasing in a concomitant fashion. Interestingly, there was not a significant correlation between sleep time and waking BAC or BREC at 03.00 hr. This may have resulted from an altered sensitivity to ethanol at this time of increased activity.

SS Mice

Figure 2 shows mean sleep times, waking BACs, and waking BRECs as a function of time of day for SS mice that received the 4.1 g/kg dose. It is interesting to note that sleep times were not found to differ significantly across time, $F(3,36)=0.9$, $p=0.43$. However, time of day did have a significant effect on both waking BAC and waking BREC: $F(3,36)=3.4$, $p<0.05$; $F(3,36)=23.0$, $p<0.001$, respectively.

As for the LS mice, multivariate analysis of variance again revealed a significant variation over time for these variables taken collectively, $F(9,83)=7.4$, $p<0.001$, and "brain ethanol concentration" was again found to be the most important variable in discriminating among time points: first discriminant function coefficient=+0.99 for BREC, −0.39 for sleep times, and −0.37 for BAC.

When Pearson product moment correlation coefficients were determined for sleep time and waking blood and brain ethanol concentrations at each point, significant correlations between sleep times and waking BRECs were found at 03.00 ($r=+.60$, $p<0.05$) and at 09.00 ($r=-.74$, $p<0.01$). No other correlations between these variables at any time point were found to be significant.

EXPERIMENT 2

The 4.1 g/kg dose administered to the SS mice resulted in sleep times ranging from a mean near 30 min to approximately 50 min. No circadian influence was detected. However, significant variations were seen for waking BAC and BREC. In view of the possibility that the SS mice given the 4.1 g/kg dose could have regained the righting response before absorption was complete, a larger ethanol dose was administered.

Figure 3 shows mean sleep times, waking BACs, and waking BRECs as a function of time of day for SS mice that received the 5.0 g/kg dose. The results of the individual one-way analyses of variance revealed no significant variations over time for any variable: sleep time, $F(3,25)=1.28$, $p=0.30$; waking BAC, $F(3,25)=2.12$, $p=0.12$; waking BREC, $F(3,25)=2.80$, $p=0.06$. The multivariate analysis of variance revealed a significant variation over time for these variables taken collectively, $F(9,56)=2.1$, $p=0.04$. "Brain ethanol concentration" and "blood alcohol concentration" were found to be equally important in discriminating among time points: first discriminant function coefficient=+0.79 for BREC, −0.79 for BAC, and −0.29 for sleep time.

Pearson product moment correlation coefficients were again determined for sleep time and waking blood and brain ethanol concentrations at each time point. Significant correlations between these parameters were found only at 09.00 hr: sleep time and waking BAC, $r=-.77$, $p<0.05$; sleep time and waking BREC, $r=-.77$, $p<0.05$; waking BAC and waking BREC, $r=+.69$, $p<0.05$. Even though the SS mice were clearly into the elimination phase of ethanol metabolism when these parameters were assessed at 15.00 and 21.00 hr, none of the correlations was significant at those times. The fact that the SS correlations were not significant, while significant correlations were found for the LS mice, may indicate a greater genetic variability in the SS animals [5].

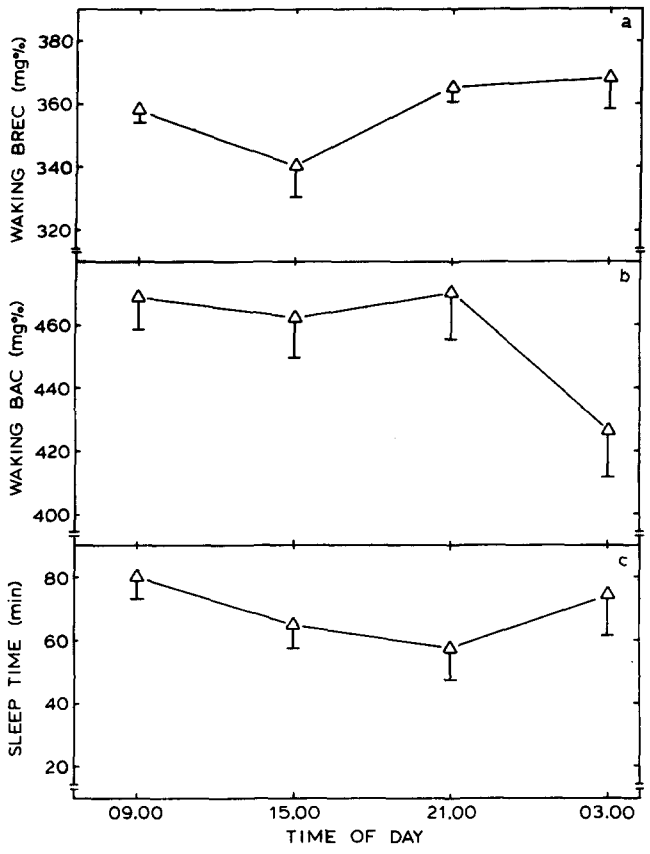

FIG. 3. Mean (a) waking brain ethanol concentrations (mg%=mg ethanol/100 g brain), (b) waking blood ethanol concentrations (mg%=mg ethanol/100 ml blood), and (c) sleep times for SS male mice injected with 5.0 g/kg ethanol at 09.00, 15.00, 21.00, or 03.00 hr. N=8 animals per group. Bars represent standard errors.

GENERAL DISCUSSION

These results provide data to document circadian and genetic influences on ethanol response. Sleep times, waking BACs, and waking BRECs showed circadian variations in LS mice, whereas SS mice failed to manifest such a pattern at the 5.0 g/kg dose. The longest sleep time in the LS mice corresponded to the lowest waking BAC, which was to be expected since the animals that sleep the longest should metabolize the most ethanol before awakening. In contrast, there was no difference in waking BREC between the shortest (09.00 hr) and the longest (15.00 hr) sleep times for LS mice. If the duration of ethanol sleep time is mediated by the central nervous system and if the concentration of ethanol in brain tissue is the critical factor in determining sleep time, then there are clear circadian variations in tissue sensitivity in the LS mice.

The SS mice failed to exhibit a circadian influence on sleep time at either the 4.1 or 5.0 g/kg dose, and the absence of circadian variations in waking BACs and BRECs at the 5.0 g/kg dose is consistent with the conclusion that CNS sensitivity to ethanol does not change during the day for SS mice. However, circadian variations in BAC and BREC were seen at the 4.1 g/kg dose. As mentioned previously, we were concerned that the SS mice given the 4.1 g/kg dose in Experiment 1 may have regained the righting response before ethanol absorption was complete. The poor correlation between BAC and BREC in the SS animals in the first experiment is consistent with that suggestion. Absorption and/or distribution may not have been complete and, as a result, an equilibrium between blood and brain concentrations may not have been achieved. Longer sleep times were attained with the 5.0 g/kg dose, and no circadian variations were observed in any of the parameters. These observations suggest circadian influences on absorption or distribution in the SS mice, but no circadian influence on CNS sensitivity.

One of our major concerns arising from these experiments was the possibility that peripheral organ toxicity resulting from a high ethanol concentration may have influenced sleep time. The recommended ethanol concentration for an IP injection is 10% w/v [34]. If a dose of 4.0 g/kg is administered in a 10% w/v concentration, the volume of injection for a 25-g mouse is 1.0 ml. This is an excessively high volume for an injection solution in a mouse and results in inconsistent absorption and distribution of the drug (Collins, unpublished observations). Consequently, a large injection volume may result in large variability in behavioral and physiological responses. A smaller injection volume necessitates a greater ethanol concentration if the same dose is to be achieved. We chose to compare the sleep-time responses of the LS and SS mice at an ethanol concentration similar to the concentration used during the selection process (23.5% w/v) [16]. In the present experiments, a 25.0% w/v ethanol solution was administered to LS and SS mice so as to give doses of 2.5 and 5.0 g/kg, respectively. Since submission of this paper, we have observed that LS mice exhibit longer sleep times and a greater hypothermic response to lower ethanol concentrations than do SS mice (Gilliam and Collins, submitted). These genetic differences in response to ethanol concentration are further evidenced by greater respiratory depression in LS than in SS mice at lower ethanol concentrations [6]. Thus, the LS mice are more responsive to changes in ethanol concentration than are the SS. It is possible that the genetic influence on circadian effects seen in this study is a result of the difference in response of LS and SS mice to high ethanol concentrations. Whatever the cause of the circadian influence, it is quite likely that genetic factors control the expression of this effect.

Differences in the rate of drug absorption and distribution (from the site of administration into the blood) are known to depend on the route of administration [3]. Also, temporal variations in the rate of drug absorption and distribution may result in variations in the concentration of drug in brain tissue. Thus, differences in brain ethanol concentrations may be a result of factors influencing the rate of absorption from the site of administration. Several of these factors (general activity, food intake, and hepatic blood flow) are known to show circadian variations [27].

Results obtained with other drugs parallel those obtained in the present experiment. Roberts *et al.* [26] found that the length of barbiturate-induced anesthesia did not show a temporal variation, whereas brain drug concentrations were significantly greater at 20.00 hr than at 08.00 hr. These results were taken to indicate temporal variation in brain sensitivity to barbiturates. In the present experiment, sleep times at 09.00 and 21.00 hr were similar within each line, but significantly higher BRECs were found for the LS mice at 21.00 hr than at 09.00 hr. This result may reflect differences in tissue sensitivity at these times of the day.

Other factors may also account for the paradoxical results

observed in this experiment. For example, circadian variations in the elimination of ethanol may be important in determining behavioral and physiological responses. We have observed circadian influence on the pharmacokinetics of ethanol in LS and SS after an IP dose of 4.1 g/kg [4]. Circadian variations in the rate of ethanol elimination from the blood have also been discussed elsewhere [10, 23, 28, 30–33, 35]. Investigators who have measured the in vitro metabolism of ethanol by alcohol dehydrogenase found that it was invariant throughout a 24-hr period [2]. However, others have found significant circadian variations in hepatic drug metabolism [17,24].

Other investigators who have measured the duration of barbiturate-induced anesthesia have found circadian variations in sleep time [17,19]. In those experiments, there was a significant negative correlation between duration of anesthesia and rate of barbiturate oxidation in the liver. Similarly, there may be a significant negative correlation between rate of ethanol oxidation in the liver and ethanol-induced sleep time. Hence, circadian variations in rate of ethanol elimination may account for some of the behavioral differences seen in the present experiment.

In conclusion, the results of the present experiment substantiate the notion that circadian variations influence ethanol's effects. In addition, the finding of such variations in one selected mouse line (LS) and not in another (SS) suggests that genetic factors may influence circadian rhythms. The responses elicited by ethanol at any time point were most influenced by brain ethanol concentration and tissue sensitivity, both of which appear to be subject to genetic influence.

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