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Circadian activity rhythms in selectively bred ethanol-preferring and nonpreferring rats

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Abstract

Chronic alcohol intake is associated with dramatic disruptions in sleep and other circadian biological rhythms in both humans and experimental animals. In human alcoholics, these disruptions persist during extended abstinence and appear to promote relapse to drinking. Whereas chronic ethanol intake alters fundamental properties of the circadian pacemaker in unselected rats, nothing is known concerning circadian pacemaker function in selectively bred ethanol-preferring and nonpreferring rats, which are the most widely accepted animal models of genetic predisposition to alcoholism. The present experiments were designed to characterize free-running circadian activity (wheel-running) rhythms under both constant darkness and constant light in selectively bred ethanol-preferring (P, HAD2) and nonpreferring (NP, LAD2) rats. Differences in circadian organization between ethanol-preferring and nonpreferring animals were seen for both pairs of selected lines (P vs. NP; HAD2 vs. LAD2), but these differences were not identical in the two line pairs. For example, although P rats showed shorter free-running periods than NP rats only in constant light, HAD2 rats showed shorter free-running periods than LAD2 rats only in constant darkness. In addition, ethanol-preferring HAD2 rats showed a high rate of rhythm ‘‘splitting’’ that was not seen in any of the other three lines. Taken together, these results suggest that the circadian pacemakers of P and NP rats differ mainly in light sensitivity, whereas those of HAD2 and LAD2 rats differ in their intrinsic period. © 2005 Elsevier Inc. All rights reserved.

Keywords: Ethanol; Preference; Rat; Circadian activity rhythm; Sleep

1. Introduction

Chronic alcoholism is associated with dramatic disruptions in sleep and other circadian biological rhythms (Brower, 2001; Fonzi et al., 1994; Kodama et al., 1988; Kuhlwein et al., 2003; Sano et al., 1993). These chronobiological alterations can persist through extended periods of abstinence (Drummond et al., 1998; Landolt & Gillin, 2001), and are associated with increased risk of relapse to drinking (Brower, 2003; Brower, 2001; Gillin et al., 1994). Further, sleep quality and other chronobiological parameters may also predict differences in initial susceptibility to alcohol abuse and alcoholism (Brower, 2001; Crum et al., 2004). Despite these extensive clinical observations, it remains unclear whether and to what extent the chronobiological disruptions seen in human alcoholics are due to alterations in the normal functioning of the circadian pacemaker, as opposed to pathophysiological processes downstream from the pacemaker (Rosenwasser, 2001).

Studies of chronic ethanol treatment in experimental animals maintained under typical light–dark (LD) entrained conditions have revealed chronobiological disturbances quite similar to those seen in human alcoholics (Ehlers & Slawecki, 2000; El-Mas & Abdul-Rahman, 2000; Kakihana & Moore, 1976; Kubota et al., 2002; Rajakrishnan et al., 1999; Rouhani et al., 1990). In addition, experiments with rats and hamsters maintained under free-running conditions (i.e., in constant light or constant darkness) suggest that chronic ethanol intake alters fundamental circadian timing processes as well as the response of the circadian pacemaker to light and possibly other entraining signals (Dwyer & Rosenwasser, 1998; Joy & Turek, 1989; Mistlberger & Nadeau, 1992; Rosenwasser et al., 2005a,b). Reciprocally, voluntary ethanol intake is increased in rats in response to experimental light–dark schedules simulating jet lag and shiftwork-induced circadian desynchronies (Gauvin et al., 1997a,b). Although the mechanisms underlying these effects have not been elucidated, several ethanol-sensitive neurotransmitters and neuromodulators, including excitatory and

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inhibitory amino acids (Faingold et al., 1998), serotonin (LeMarquand et al., 1994), and neuropeptide Y (Clark et al., 1998; Thiele & Badia-Elder, 2003), are known to also be critically involved in circadian pacemaker regulation (Rosenwasser, 2003). In addition, mutation of the circadian ‘‘clock gene’’, *Per2*, is associated with increased ethanol intake in mice (Spanagel et al., 2005), whereas adult and perinatal ethanol treatments alter the expression of neuropeptides (Clark et al., 1998; Madeira et al., 1997; Madeira & Paula-Barbosa, 1999), growth factors (Allen et al., 2004), and circadian clock genes (Chen et al., 2004) and promote glial proliferation (Satriotomo et al., 1999) in the hypothalamic suprachiasmatic nucleus (SCN), site of the primary circadian pacemaker in mammals (Rosenwasser, 2003).

Although these studies may be relevant to understanding the chronobiology of alcoholism, they have not used explicit, validated animal models of the human disease. For example, selectively bred ethanol-preferring and nonpreferring rat lines provide widely accepted animal models of genetic predisposition to excessive alcohol intake and alcoholism, and ethanol-naïve animals from selected high-preference and low-preference lines exhibit numerous behavioral, physiological, and neurochemical differences linked potentially to the ethanol-preference phenotype (Murphy et al., 2002; Rodd et al., 2004). Both unselected rats and selectively bred ethanol-preferring rats exhibit nocturnal patterns of ethanol intake that closely resemble the normal daily variation of food and water intake when maintained under standard LD cycles (Hiller-Sturmhofel & Kulkosky, 2001). On the other hand, nothing is known concerning the properties of the free-running circadian pacemaker, expressed under conditions of constant darkness (DD) or constant light (LL), in ethanol-preferring and nonpreferring rats. To determine whether genetic ethanol preference is associated with alterations in circadian pacemaker function, we have examined the expression of circadian running-wheel activity rhythms in ethanol-naïve animals of the ethanol-preferring P and HAD2 rat lines, and the corresponding ethanol nonpreferring NP and LAD2 lines, developed at the Indiana University (Murphy et al., 2002). These lines comprise two of the three pairs of selectively bred lines developed at Indiana (the third being the HAD1 and LAD1 lines), and were derived from very different progenitor stock via independent breeding programs. Of the various ethanol-preferring rat lines that have been developed in different laboratories, the Indiana lines arguably provide the most extensively validated animal models of genetic predisposition to alcoholism (Murphy et al., 2002; Rodd et al., 2004).

2. Materials and methods

2.1. Subjects and apparatus

2.1.1. Experiments 1A and 1B

Male P ($N = 5$) and NP ($N = 6$) rats (55th generation) of approximately six weeks of age were obtained from the Indiana University Alcohol Research Center and maintained in running wheel cages (wheel diameter: 35 cm) equipped with standard side compartments (Lafayette Instruments). Food (Prolab RMH 3000) and water were always freely available. The running wheel cages were housed within light- and sound-shielded enclosures, and the enclosures were equipped with standard incandescent light fixtures that could be controlled by computer. Wheel revolutions were detected via microswitches, monitored by a personal computer running the Dataquest III interface system (MiniMitter Co.), and stored in 10-min bins for subsequent statistical analysis, as described below.

2.1.2. Experiment 2

Male P ($N = 4$), NP ($N = 5$), HAD2 ($N = 5$), and LAD2 ($N = 6$) rats (P, NP: 56th generation; HAD2, LAD2: 43rd generation) of approximately 6 weeks of age were obtained from the Indiana University Alcohol Research Center and maintained in Nalge running wheel cages (wheel diameter: 35 cm) with cut-away bottoms and wire mesh floors (MiniMitter Co.). Food (Prolab RMH 3000) and water were always freely available. The running wheel cages were housed within light- and sound-shielded enclosures, and the enclosures were equipped with fluorescent light fixtures that could be controlled by computer. Wheel revolutions were detected via microswitches, monitored by a personal computer running the ClockLab interface system (Actimetrics Co.), and stored in 6-min bins for subsequent statistical analysis, as described below.

2.2. Procedures

2.2.1. Experiment 1A

The animals were first maintained under free-running conditions in constant darkness (DD) for 28 days, and then under constant light (LL; about 30 lux) for an additional 31 days.

2.2.2. Experiment 1B

Next, an LD 12:12 schedule was imposed, and once all animals had stably entrained to the LD 12:12 cycle (21 days), the period of the LD cycle (T) was gradually shortened, while maintaining the L/D ratio at 50% ($T = 23.8$ h, LD 11.9:11.9, 15 days; $T = 23.6$, LD 11.8:11.8, 8 days; $T = 23.4$, LD 11.7:11.7, 5 days; $T = 23.2$, 11.6:11.6, 9 days). Finally, the animals were maintained under $T = 23.0$ (LD 11.5:11.5) for an additional 32 days.

2.2.3. Experiment 2

The animals were initially maintained in DD for 23 days, followed by 82 days in LL (about 50 lux), and then returned to DD for an additional 44 days.

The experimental procedures described in this report were reviewed and approved by the University of Maine Institutional Animal Care and Use Committee.

2.3. Data analysis and statistics

2.3.1. Experiment 1A

Raster-style actograms were generated using Dataquest III software. In addition, three quantitative parameters, representing the period, amplitude, and level of the free-running activity rhythm, were derived for each animal for selected data samples representing the final two weeks of each lighting condition (DD, LL). Following the suggestion of Levine et al. (2002), free-running circadian periods were estimated with several independent and arithmetically dissimilar approaches. Thus, using routines supplied within the ClockLab circadian analysis package, free-running periods were estimated by (1) computer-assisted visual-graphic analysis based on fitting linear regression functions to activity onsets over successive days; (2) chi-square periodogram analysis, a nonparametric (i.e., waveform-independent) approach that estimates the period and amplitude of periodic processes in a time series (Sokolove & Bushell, 1978); and (3) Lomb–Scargle periodogram analysis, a cosine-based approach that estimates the period and strength (power) of multiple periodic components in time-series data (Ruf, 1999; Van Dongen et al., 1999). Preliminary analyses showed that all three measures of free-running period were strongly correlated with one another, and that the statistical conclusions presented in this paper were essentially independent of the method of period estimation. Therefore, to minimize variance, the free-running period values reported in this paper represent the arithmetic mean of the three independent period estimates for each data sample. In addition, the amplitude (Q_p) of the dominant circadian peak in the chi-square periodogram was examined as a measure of the ‘‘robustness’’ of the activity rhythm (Levine et al., 2002), and the number of wheel-turns per day examined as a measure of activity level. Because activity level data are expected to be strongly skewed, this variable was log transformed for statistical analysis to normalize distributions.

Each of the three dependent measures (period, amplitude, and log activity) was subjected to a separate 2×2 mixed factorial analysis of variance (ANOVA), with lighting condition (DD, LL) as a within-subjects factor and selected line (P, NP) as a between-groups factor, followed by post hoc pairwise tests (alpha = 0.05) for simple effects of both line and lighting condition.

2.3.2. Experiment 1B

Raster-style actograms were generated using ClockLab software. The stability of entrainment and the occurrence of ‘‘relative coordination’’ and associated ‘‘phase-jumps’’ under the different LD cycles were evaluated initially by visual inspection of the actograms. (*Relative coordination* refers to a condition of unstable, partial, or temporary circadian entrainment occurring under a marginally effective entrainment stimulus. When maintained under a marginally effective entrainment cycle, circadian rhythms may periodically show abrupt and very large amplitude phase shifts, i.e., *phase-jumps*, that take the system from one quasisteady state to the next; cf., Pittendrigh, 1981a). In this study, phase-jumps could be recognized unambiguously as rapid 24-h phase displacements in which the active phase of the circadian cycle shifted abruptly (within two to three cycles) from one dark phase to the next (cf., Fig. 4). Based on visual inspection, the proportion of animals in the two lines showing phase-jumps was compared using a chi-square test, and the mean number of phase-jumps per animal compared with a *t* test. Given the relatively short exposures to each LD cycle, coupled with the well-known tendency for circadian phase to progressively delay as LD cycle period is shortened (Aschoff, 1981; Pittendrigh, 1981a), it was not practical to evaluate the phase angle of entrainment under the different LD cycles. Instead, estimates of free-running period were obtained graphically for each animal under each LD cycle using ClockLab. In this way, differences between the periods of the LD cycle and activity rhythm could be used as a quantitative index of the effectiveness of entrainment (i.e., larger differences imply less effective entrainment).

2.3.3. Experiment 2

Free-running periods, chi-square periodogram amplitudes, and daily activity levels were determined for each animal over the final three weeks of each lighting condition (DD1, LL, DD2), except as follows. Because most (3/5) HAD2 rats showed rhythm ‘‘splitting’’ under LL (see Section 3), free-running periods and periodogram amplitudes for these three animals were derived from the initial weeks of LL, prior to the onset of splitting. These values were subjected to a three-factor mixed ANOVA, using the factors ‘‘ethanol preference’’ (P + HAD2 vs. NP + LAD2), ‘‘line pair’’ (P/NP vs. HAD2/LAD2), and lighting condition (DD1, LL, DD2). This approach allowed for the detection of (1) statistical main effects due to ethanol preference as expressed across the two line pairs, (2) statistical differences between the two line pairs, regardless of ethanol preference, and (3) possible interactions between these factors and lighting conditions. Three-factor ANOVAs were followed by separate two-factor ANOVAs (condition and, ethanol preference) for each line pair, and by planned pairwise comparisons between the two lines in each line pair, within each lighting condition.

In addition, the possible presence of multiple periodic components contributing to the spectral composition of the free-running activity rhythm was assessed via the Lomb–Scargle periodogram, performed on a range of test periods between 6 and 30 h. These power spectra were first computed separately for each animal in each condition, and then averaged across animals within lines and conditions. Finally, circadian waveforms under DD1 conditions were constructed for each animal by averaging activity count data across days in time bins of one ‘‘circadian hour’’ (by convention, one circadian hour is

equal to 1/24th of the free-running period). These individual profiles were then normalized proportionate to their means, and then averaged across animals by aligning each profile to a phase determined by the first hourly total below the daily mean, which was defined as "circadian time" zero (CT 0), marking the end of the active phase of the cycle.

3. Results

3.1. Experiment 1A

All animals from both selected lines exhibited coherent and well-organized activity rhythms under both DD and LL conditions, and as expected for nocturnal animals such as the rat, free-running periods were clearly longer under LL conditions than under DD (Fig. 1). Whereas free-running periods appeared similar in the two lines under DD, visual inspection of the actograms suggested that P rats displayed shorter free-running periods than did NP rats under LL. This impression was confirmed statistically by ANOVA, which revealed a significant light condition by line interaction [$F(1, 9) = 5.30$, $P = .047$], as well as significant main effects of both light [$F(1, 9) = 90.76$, $P < .001$] and line [$F(1, 9) = 10.17$, $P = .011$] (Fig. 2). The light by line interaction was explored further using pairwise comparisons, which revealed significant light effects on free-running period for both lines, whereas significant line differences were seen only under LL and not under DD conditions (Fig. 2).

Whereas chi-square periodogram amplitudes were slightly higher in P than in NP rats under DD, amplitudes were essentially identical under LL (Fig. 2). Two-way ANOVA detected only a significant effect of lighting condition [$F(1, 9) = 30.40$, $P < .001$], indicating that amplitudes were significantly lower under LL than under DD, but no main effect of line [$F(1, 9) = .17$, $P = .69$] or any line by condition interaction [$F(1, 9) = .68$, $P = .43$] was seen.

P rats also showed somewhat lower levels of running-wheel activity than did NP rats, both in DD and in LL (Fig. 2). Nevertheless, ANOVA revealed only a significant main effect of light condition [$F(1, 9) = 25.12$, $P = .001$], indicative of reduced activity in LL relative to DD, but no main effect of line [$F(1, 9) = 2.84$, $P = .13$] or any lighting by line interaction [$F(1, 9) = .23$, $P = .64$]. Despite the lack of significant line effects in the ANOVA, exploratory pairwise comparisons showed a near-significant line difference in activity levels in DD [$t(9) = 2.08$, $P = .067$], but not in LL.

3.2. Experiment 1B

All rats of both lines expressed normally entrained activity rhythms under LD 12:12 (Fig. 3). As the period of the LD cycle was shortened, the phase relationship of activity onset to the onset of darkness (i.e., the phase angle) delayed progressively in both P and NP lines for LD cycle periods between 24.0 (LD 12:12) and 23.4 (LD 11.7:11.7) (Figs. 3 and 4). Progressive phase delay is the expected response of entrained circadian systems to short-period entraining stimuli, and has been demonstrated repeatedly in many species (Aschoff, 1981; Pittendrigh, 1981a), including the rat (Stephan, 1983). Extensive prior research

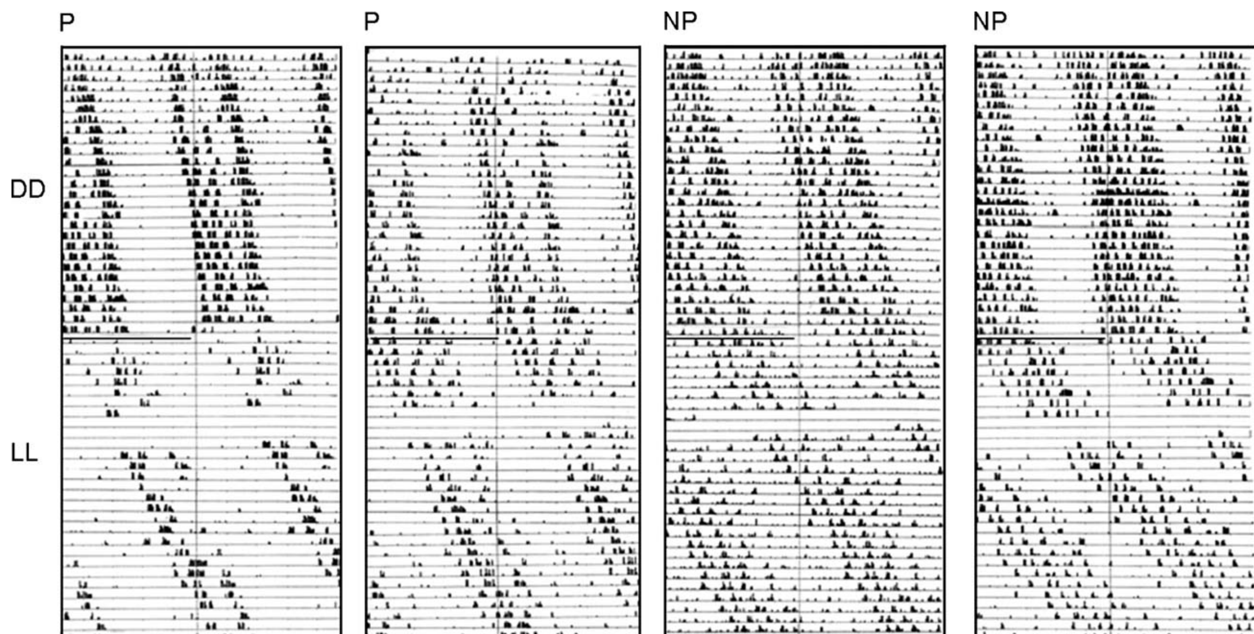

Fig. 1. Double-plotted (48-h span) raster-style actograms showing running-wheel activity rhythms under constant darkness (DD) and constant light (LL) in two representative animals of each line (ethanol-preferring, P; nonpreferring, NP) (Experiment 1A). The 2–3 day blank space in each record is due to a recording failure.

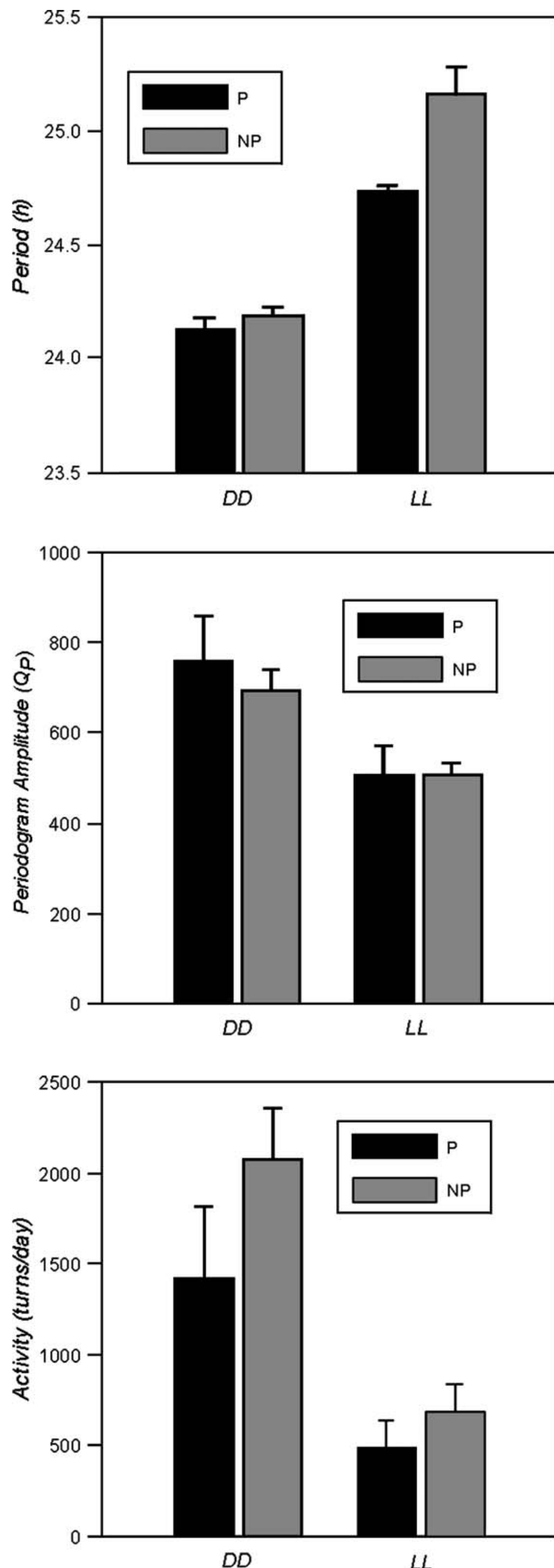

Fig. 2. Mean (+S.E.M) free-running period (top), periodogram amplitude (middle), and activity level (bottom) in ethanol-preferring (P) and nonpreferring (NP) rats under constant darkness (DD) and constant light (LL) (Experiment 1A); see text for computational and statistical details.

also demonstrates that circadian systems entrain effectively only to entraining stimuli within a restricted range of periods, and that the precise upper and lower bounds of the effective range depend on a variety of organismic and environmental factors (Pittendrigh, 1981a). In this experiment, a clear line difference was apparent in the lower limit of the range of entrainment, in that NP rats generally entrained successfully under the 23-h LD cycle (LD 11.5:11.5), whereas P rats generally failed to entrain to this LD cycle, and instead expressed a repeating pattern of period-modulation and abrupt ''phase-jumps'', known as relative coordination (Pittendrigh, 1981a) (Figs. 3 and 4). Although the proportion of animals exhibiting phase-jumps did not differ significantly between the two lines (Fig. 4) [chi-squared, $P = .12$], P rats showed marginally more phase-jumps per animal than did NP rats (Fig. 4) [$t(9) = 2.11$, $P = .06$].

3.3. Experiment 2

Animals from all four selected lines showed clear free-running circadian activity rhythms under both DD and LL conditions, and as expected, free-running periods were consistently longer in LL than in DD (Fig. 5). In addition, most (3/5) of the HAD2 rats showed ''splitting'' of the nocturnal activity band into two discrete components under LL, a phenomenon that was not observed in any of the other lines (Fig. 5). Although splitting of circadian activity rhythms has been observed previously in rats (Boulos & Terman, 1979), this phenomenon is considered to be relatively rare in this species (Rosenwasser & Adler, 1986) and is reported much more frequently in other species (among rodents, most notably in the Syrian hamster; Pittendrigh, 1981b; Pickard et al., 1993; Rosenwasser & Adler, 1986). Because splitting is thought to reflect LL-induced uncoupling between multiple underlying circadian oscillators, these observations suggest an important difference in the internal organization of the HAD2 and LAD2 pacemakers that is not apparently seen in the P and NP lines.

Three-factor ANOVA conducted on free-running periods revealed significant main effects of lighting condition (DD1, LL, DD2) [$F(2, 32) = 236.89$, $P < .001$], ethanol preference (P + HAD2 vs. NP + LAD2) [$F(1, 16) = 31.83$, $P = .001$], and line pair (P + NP vs. HAD2 + LAD2) [$F(1, 16) = 29.60$, $P < .001$] (Fig. 6). These main effects indicate that, overall, free-running periods were shorter in DD than in LL, shorter in ethanol-preferring (P, HAD2) than in nonpreferring (NP, LAD2) lines, and shorter in the P/NP line pair than in the HAD2/LAD2 line pair.

In addition to these main effects, ANOVA also detected a significant two-way ethanol preference by line pair interaction [$F(1, 16) = 5.58$, $P = .031$]. This interaction was explored further by conducting separate two-factor (condition by ethanol preference) ANOVAs for each line pair. For the P/NP line pair, significant effects were seen for

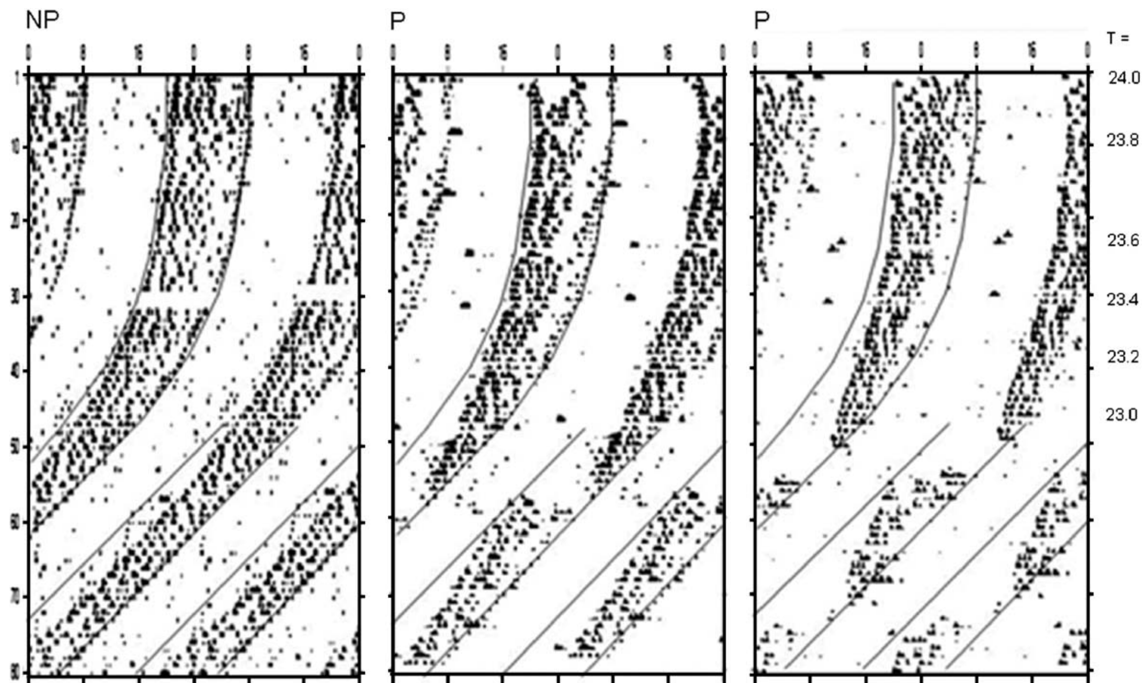

Fig. 3. Double-plotted (48-h span) raster-style actograms showing wheel-running activity patterns under gradually shortening light–dark (LD) cycles, for one representative ethanol-nonpreferring (NP) and two representative ethanol-preferring (P) rats (Experiment 1B). Oblique lines in the body of the records show the timing of the dark phase of the LD cycle, and changing LD cycle period (T) is indicated to the right. Note the failure of P rats to exhibit stable entrainment under $T = 23$ h.

lighting condition [$F(2, 14) = 224.38$, $P < .001$] and ethanol preference [$F(1, 7) = 20.72$, $P = .003$], and for the condition by preference interaction [$F(2, 14) = 5.78$, $P = .015$]. Thus, whereas free-running periods were generally shorter in P than in NP rats, this difference was due mainly to the LL condition. This conclusion was confirmed by separate pairwise comparisons between P and NP rats for each lighting condition, which revealed a significant line difference only in LL [$t(7) = 3.65$, $P = .008$], but not in DD1 or DD2.

For the HAD2/LAD2 line pair, significant effects were seen for lighting condition [$F(2, 18) = 103.32$, $P < .001$] and ethanol preference [$F(1, 9) = 22.37$, $P = .001$], but the condition by line interaction was not significant [$F(2, 18) = 0.18$, $P = .83$]. These results indicate that HAD2 rats showed generally shorter free-running periods than LAD2 rats, across lighting conditions. Nevertheless, despite the lack of a condition by preference interaction for this line pair, pairwise comparisons within lighting conditions revealed significant differences between the HAD2 and LAD2 lines only in DD [DD1: $t(9) = 2.62$, $P = .028$; DD2: $t(9) = 3.48$, $P = .007$], but not in LL.

Three-factor ANOVA conducted on periodogram amplitudes revealed significant main effects of lighting condition [$F(2, 32) = 18.57, P < .001$] and line pair [$F(1, 16) = 14.64$, $P = .001$], but no main effect or any interaction involving ethanol preference (Fig. 6). These results indicate that, overall, amplitudes declined gradually over the course of the experiment, and that animals of the P/NP line pair displayed lower amplitudes than did those of the HAD2/LAD2 line pair. Despite the lack of a significant three-way interaction, separate two-factor ANOVAs were conducted for each line pair as an exploratory analysis. For the P/NP line pair, significant effects were seen for both lighting condition [$F(2, 14) = 53.01$, $P < .001$] and ethanol preference [$F(1, 7) = 5.96$, $P = .045$], but for the HAD2/LAD2 line pair, only the lighting-condition effect was significant [$F(2, 18) = 4.91$, $P = .021$]. These results indicate that P rats displayed greater periodogram amplitudes than did NP rats across conditions, whereas HAD2 and LAD2 animals did not differ in any condition. Confirming this suggestion, P rats showed greater amplitudes than did NP rats in both LL [$t(7) = 3.34$, $P = .013$] and DD2 [$t(7) = 3.12$, $P = .017$], but no pairwise differences were seen between HAD2 and LAD2 animals.

Three-factor ANOVA conducted on log activity levels revealed significant main effects of lighting condition [$F(2, 32) = 20.27$, $P < .001$] and ethanol preference [$F(1, 16) = 5.90$, $P = .027$], and a marginally significant effect of line pair [$F(1, 16) = 4.20$, $P = .057$] (Fig. 6). These results indicate that, overall, activity levels declined over the course of the experiment, ethanol-preferring animals showed lower activity levels than did nonpreferring animals, and animals of the P/NP line pair showed lower activity than did those of the HAD2/LAD2 line pair. In addition, a significant two-way interaction between condition and line pair [$F(2, 32) = 3.58$, $P = .040$] revealed that the decline in activity over the course of

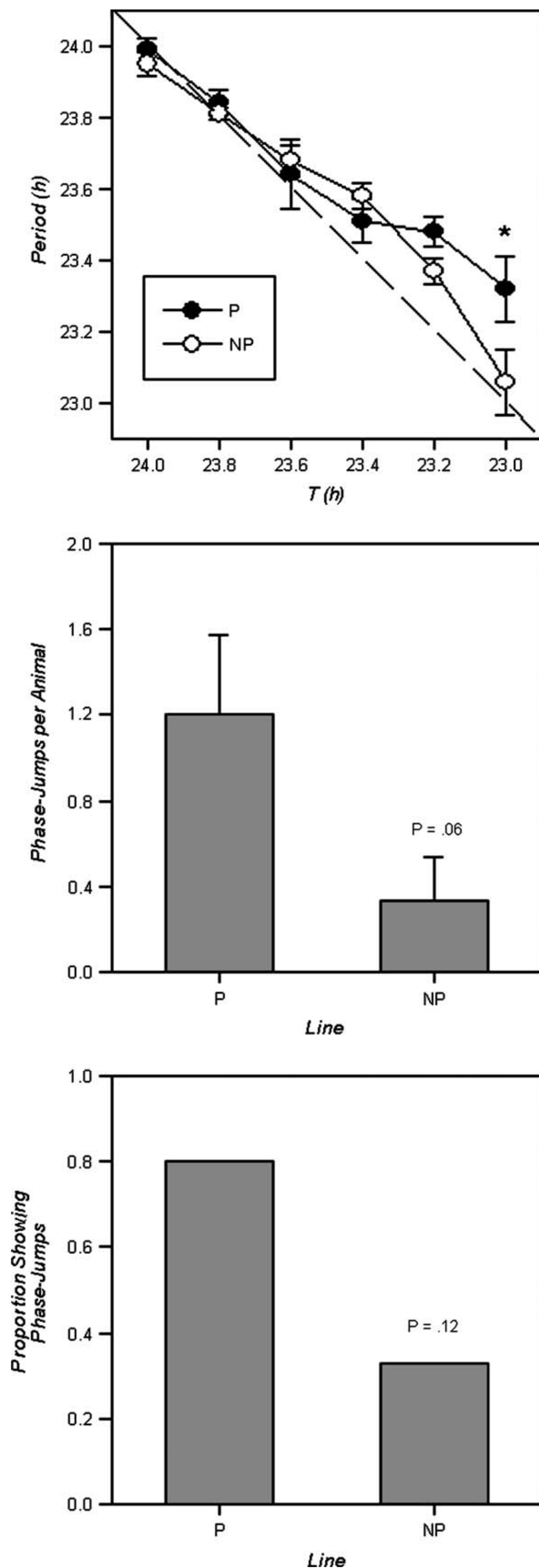

Fig. 4. (Top) Rhythm period as a function of light–dark (LD) cycle period (T) for ethanol-preferring (P) and nonpreferring (NP) rats (Experiment 1B). Asterisk indicates a significant line difference at $T = 23.0$. (Middle) Mean (+S.E.M) number of phase-jumps per animal under LD 23.0 in the two selected lines. (Bottom) Proportion of animals in each line showing at least one phase-jump under LD 23.0.

the experiment occurred mainly in animals of the P/NP line pair.

Again, despite the absence of a significant three-way interaction, two-factor ANOVAs were conducted separately for each line pair. For the P/NP line pair, significant effects were seen for both condition [$F(2, 14) = 51.22$, $P < .001$] and the condition by preference interaction [$F(2, 14) = 4.11$, $P = .040$], confirming that activity levels declined over the experiment in this line pair and indicating that the decline in activity occurred more dramatically in the NP than in the P line. These conclusions are supported further by pairwise comparisons, which showed a marginally significant difference in activity between P and NP rats in DD1 [$t(7) = 2.21$, $P = .062$], but no differences in either LL or DD2. For the HAD2/LAD2 line pair, significant main effects were seen for condition [$F(2, 18) = 5.54$, $P = .013$] and ethanol preference [$F(1, 9) = 5.66$, $P = .041$], confirming a slight decline in activity levels in this line pair, and indicating that HAD2 rats showed consistently lower activity levels than did LAD2 rats, across conditions. In further support of this conclusion, pairwise comparisons showed significant or marginal differences between HAD2 and LAD2 animals in DD1 [$t(9) = 2.41$, $P = .039$] and LL [$t(9) = 2.22$, $P = .054$] (but not in DD2).

Fig. 7 shows averaged power spectra based on the Lomb–Scargle periodogram for each line under the three conditions of the experiment. In general, animals of all four lines exhibited a dominant spectral peak at the circadian ($\approx$24-h) fundamental, as well as weaker spectral peaks at one or more harmonic frequencies (i.e., at $\approx$12, 8, or 6 h periods). Across conditions, averaged spectra were essentially identical between the two lines within each line pair (P vs. NP, HAD2 vs. LAD2), but across line pairs, animals of the P/NP line pair showed generally less spectral power, indicative of somewhat weaker rhythmicity, relative to the HAD2/LAD2 line pair. Spectral analysis also confirmed the presence of rhythm splitting in several of the HAD2 animals under LL – for these animals, power spectra revealed a dominant peak at $\approx$12 h, and little or no spectral power within the circadian frequency range.

Analysis of averaged circadian waveforms under DD1 conditions also suggested more consistent differences between the line pairs than between ethanol-preferring and nonpreferring lines (Fig. 8). Thus, P and NP rats showed very similar waveforms, characterized by clear ‘‘evening’’ and ‘‘morning’’ activity peaks, with generally lower activity occurring during the middle subjective night. These bimodal activity patterns are also apparent in the actograms presented in Fig. 5. In contrast, HAD2 and LAD2 animals showed less evidence for bimodal activity patterns (see also Fig. 5). Further, most HAD2 animals exhibited a very strong morning activity peak, with little or no evidence of an evening peak, whereas most LAD2 animals showed a strong evening peak, with little or no evidence for a discrete morning peak.

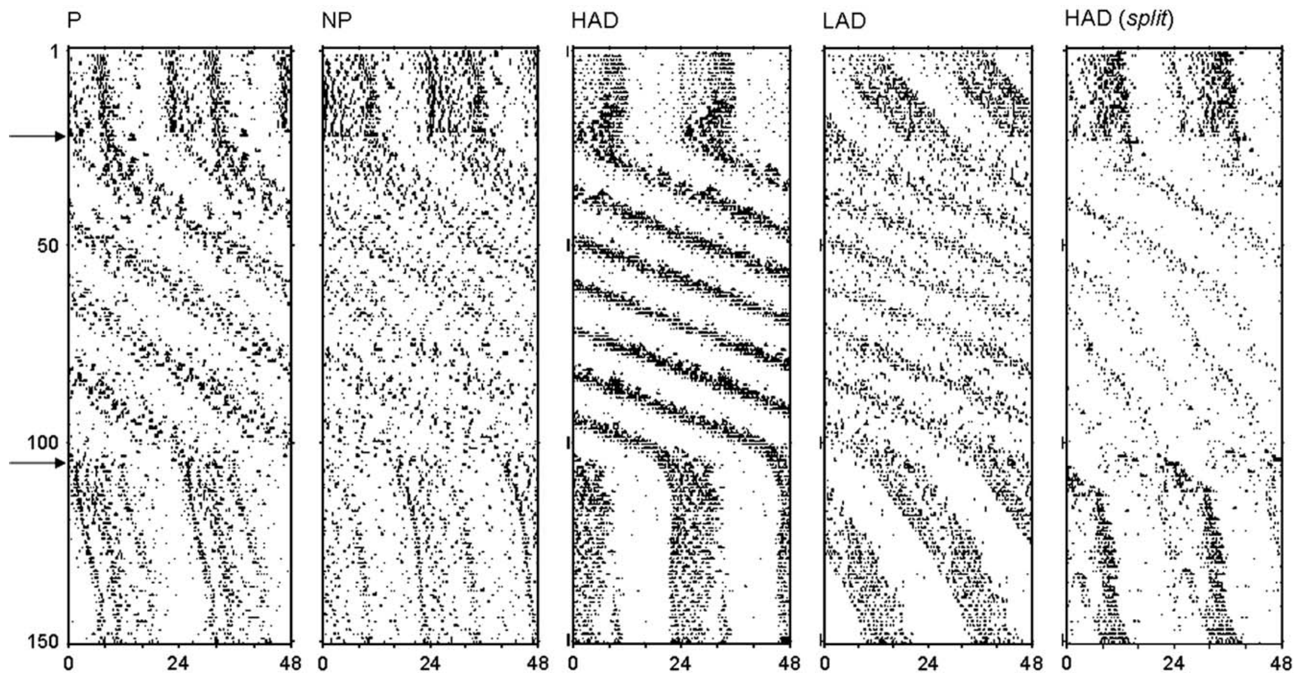

Fig. 5. Double-plotted (48-h span) raster-style actograms showing running-wheel activity rhythms under constant darkness (DD1), constant light (LL), and constant darkness (DD2) in one representative animal of each line (ethanol-preferring P, HAD2; nonpreferring, NP, LAD2), and in one additional HAD2 animal showing splitting under LL (Experiment 2). Arrows in the left margin indicate the first and last days of LL conditions.

4. Discussion

The results presented here demonstrate that selective breeding for high and low ethanol preference has resulted in differences in circadian organization in two similar but entirely independent selection programs (Murphy et al., 2002). The occurrence of different circadian phenotypes in both the P/NP and the HAD2/LAD2 line pairs suggests strongly that either genetic and/or physiological linkages exist between the mechanisms underlying the expression of ethanol preference and circadian activity rhythms. Because some of the variables we examined, including free-running period (Experiments 1A and 2) and phase changes as a function of entrainment cycle period (Experiment 1B), are considered to reflect quantitative parameters of the underlying circadian pacemaker (Pittendrigh, 1981a,b), these results indicate that fundamental circadian pacemaker properties have been coselected along with differential ethanol preference in these selected lines. This hypothesis is strengthened further by a recent report of differences in free-running circadian period between selectively bred high- and low-ethanol–preferring mice (Hofstetter et al., 2003). Indeed, the ethanol-preferring mice, like the ethanol-preferring rats in this report, displayed generally shorter free-running periods than did the corresponding nonpreferring line. Nevertheless, despite these consistencies, the exact nature of the observed differences in circadian pacemaker function between the ethanol-preferring and nonpreferring animals in each line pair was not identical. In addition, several consistent differences in circadian organization were detected between the P/NP and HAD2/LAD2 line pairs, independent of ethanol preference. Presumably, these differences reflect the different foundational stocks that were used to generate the two line pairs (Murphy et al., 2002).

In the P/NP line pair, ethanol-preferring P rats showed shorter free-running periods than did nonpreferring NP rats, but only in LL, and not in DD; this result was replicated in two independent experiments (Experiments 1A and 2). Although free-running circadian period in DD is generally taken as a direct measure of the endogenous period of the circadian pacemaker, free-running period as expressed in LL is a function of light intensity and thus reflects the sensitivity of the circadian pacemaker to light (Aschoff, 1981; Pittendrigh, 1981a,b). These observations therefore suggest that the circadian pacemaker in P rats is less sensitive to light than that in NP rats. This hypothesis is supported further by the results of Experiment 1B, which showed that P rats are less likely to exhibit stable entrainment to a short 23-h LD cycle. Because P rats showed both reduced *period-lengthening* under LL as well as impaired *period-shortening* under the short LD cycle, these results, taken together, are consistent with a generalized reduction in circadian light sensitivity, rather than a line difference in endogenous pacemaker period.

In contrast to the results with P and NP rats, ethanol-preferring HAD2 rats showed shorter free-running periods than did nonpreferring LAD2 rats even when housed in DD (Experiment 2). This finding indicates that the HAD2 and

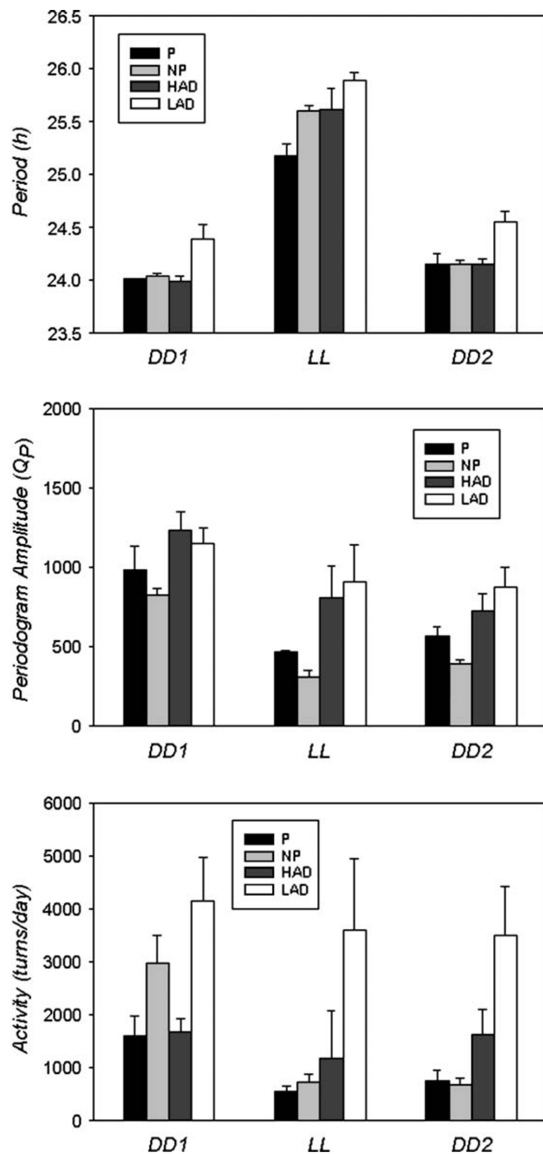

Fig. 6. Mean (+S.E.M) free-running period (top), periodogram amplitude (middle), and activity level (bottom) in ethanol-preferring (P), non-preferring (NP), HAD2, and LAD2 rats under constant darkness (DD1), constant light (LL), and constant darkness (DD2) conditions (Experiment 2); see text for computational and statistical details.

LAD2 circadian pacemakers differ in their intrinsic frequency, independent of photic stimulation. Although HAD2 and LAD2 rats did not exhibit line differences in free-running period under LL, the line differences seen in DD complicate the interpretation of LL periods, and further research would thus be required to determine whether the circadian pacemaker also exhibits differential light sensitivity in the HAD2 and LAD2 lines.

P and NP rats displayed chi-square periodogram amplitudes that were generally lower in LL than in DD in both Experiments 1A and 2. In Experiment 1A, P rats displayed slightly higher amplitudes than did NP rats, but this difference was seen only in DD and was not significant in either lighting condition. In Experiment 2, however, P rats displayed significantly higher amplitudes across DD and LL lighting conditions. Taken together, these results indicate that P rats display somewhat higher amplitude free-running rhythms than do NP rats. In contrast, HAD2 and LAD2 rats displayed periodogram amplitudes that did not differ across lighting conditions or between lines, but were significantly higher than those seen in the P/NP line pair (Experiment 2). Although periodogram amplitudes differed across lines and lighting conditions, no consistent differences as a function of ethanol preference per se were detected.

A similar conclusion may be drawn from the results of the power spectral and waveform analyses performed on the data from Experiment 2. Although P and NP rats showed very similar DD waveforms and very similar spectral profiles in both DD and LL conditions, HAD2 rats showed an unusually prominent late-night activity peak in DD and a high incidence of rhythm splitting in LL that were not seen in any of the other lines. These differences are therefore unlikely to be associated with ethanol preference, and are instead more likely to have emerged fortuitously in the course of selection.

P and NP rats showed lower activity under LL than under initial DD conditions in both Experiments 1A and 2, but in Experiment 2, activity levels failed to recover to baseline levels when the animals were returned to DD conditions following exposure to LL. Thus, the reduction in activity may have been due mainly to the passage of time, rather than changed lighting conditions per se. P rats also showed somewhat lower activity levels than did NP rats in both initial DD and subsequent LL conditions (Experiments 1A and 2), but not when returned to DD in Experiment 2. These differences were (marginally) significant only under the initial DD conditions, but could have been obscured by the generally reduced activity levels seen in both P and NP lines in later stages of the experiments. This hypothesis is supported by observations of animals of the HAD2/LAD2 line pair: unlike P/NP rats, HAD2 and LAD2 rats showed little or no reduction in activity levels over time, and HAD2 rats showed consistently lower activity levels than did LAD2 rats, across conditions.

To summarize, both P and HAD2 rats displayed shorter free-running periods than did the corresponding NP and LAD2 rats, but these results were dependent on lighting conditions, suggesting that the P rat circadian pacemaker differs from the NP rat pacemaker mainly in its light sensitivity, whereas the HAD2 rat circadian pacemaker differs from the LAD2 pacemaker mainly in its intrinsic frequency. A recent study of selectively bred ethanol-preferring and nonpreferring mouse lines showed shorter free-running periods in the ethanol-preferring line when maintained in DD (Hofstetter et al., 2003), as was seen here for HAD2 and LAD2 rats, but because the mouse lines were not tested in LL, no conclusion may be drawn regarding their circadian light sensitivity. The hypothesized difference in light sensitivity between the P and NP circadian

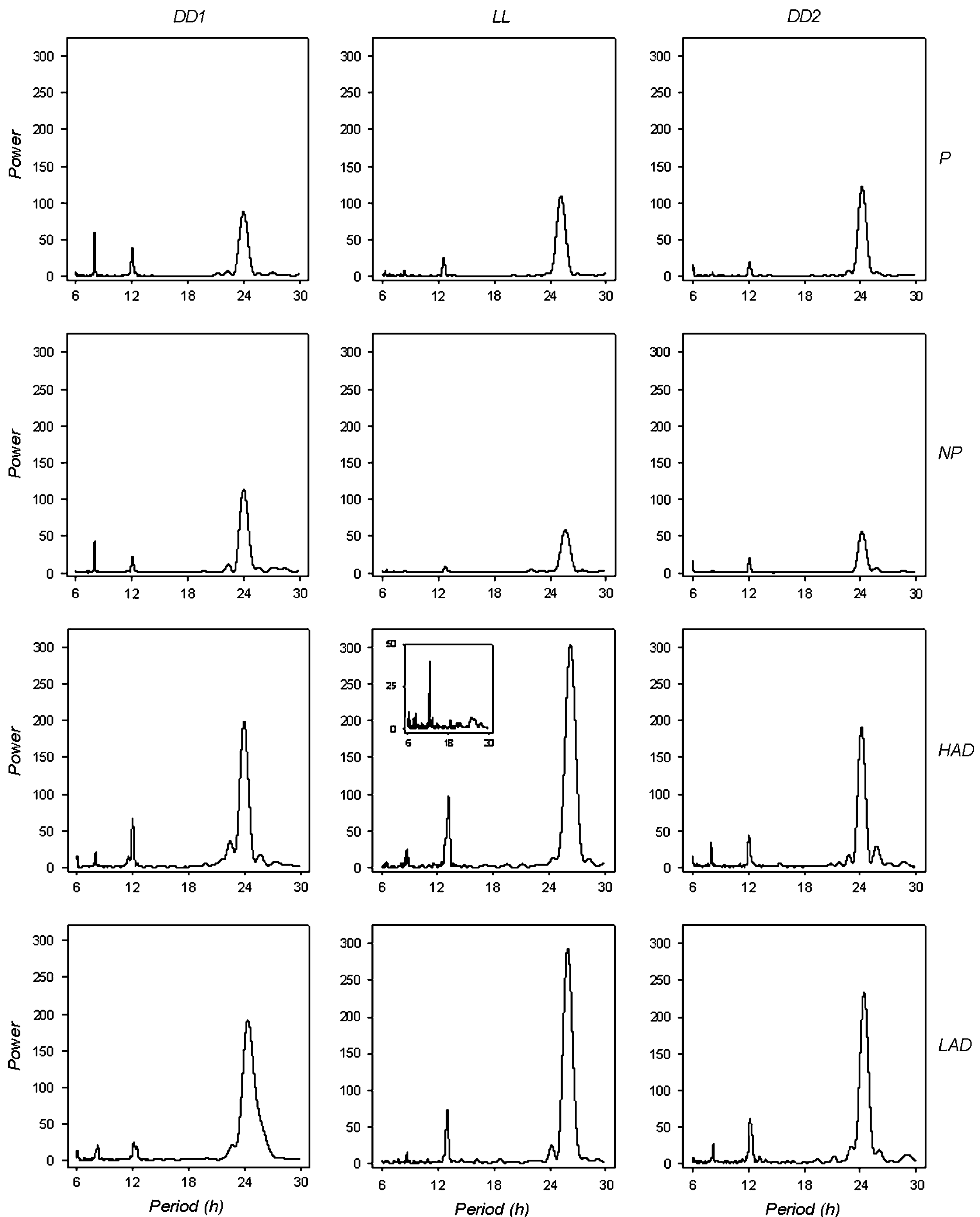

Fig. 7. Mean power spectra (Lomb–Scargle procedure) for ethanol-preferring (P), nonpreferring (NP), HAD2, and LAD2 rats under constant darkness (DD1), constant light (LL), and constant darkness (DD2) conditions (Experiment 2); for HAD2 animals under LL, separate spectra are shown for the split (inset) and unsplit animals.

pacemakers was supported further by a preliminary experiment showing impaired entrainment to a short (23-h) LD cycle in P rats, but much more work would be required to fully characterize circadian entrainment dynamics in these selected lines. Analysis of periodogram amplitudes, power spectra, and circadian waveforms revealed several

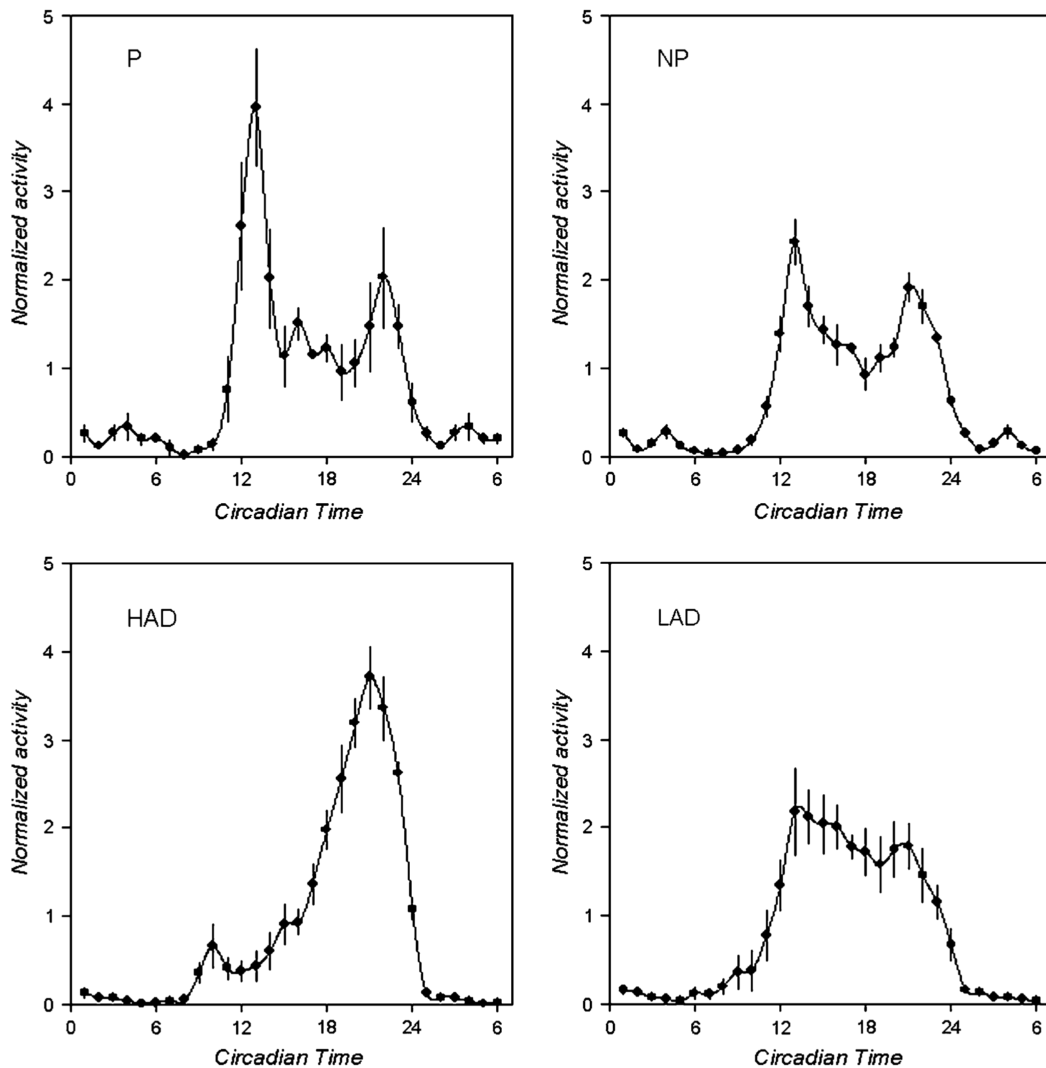

Fig. 8. Mean (±S.E.M) circadian waveforms for ethanol-preferring (P), nonpreferring (NP), HAD2, and LAD2 rats under initial constant darkness (DD1) conditions. Each *Y*-axis is normalized to its own mean level (1.0), so line differences in absolute activity level cannot be determined from these graphs. For other details, see text.

differences between the selected lines and across experimental conditions, but no clear differences related to ethanol preference per se were detected in these analyses. Finally, ethanol-preferring animals of both line pairs (P, HAD2) showed consistently lower activity levels in the initial DD conditions than did the corresponding nonpreferring lines (NP, LAD2). Under subsequent LL (and DD2) conditions, HAD2 rats continued to show lower activity than did LAD2 rats, but possible differences between P and NP rats may have been obscured by the reduced activity levels seen in both lines.

Although the genetic and/or physiological mechanisms linking chronobiological differences to differences in ethanol preference remain to be elucidated, a number of possibilities may be identified. Thus, several neurotransmitter systems that have been implicated in the neurobehavioral effects of ethanol, including ethanol preference, are known to also be involved critically in circadian pacemaker regulation. For example, selectively bred ethanol-preferring rat lines, including both P and HAD (HAD1) rats, may be characterized by a generalized upregulation of GABAergic and downregulation of serotonergic neurotransmission, especially within limbic and basal forebrain areas, relative to nonpreferring lines (Murphy et al., 2002). Although the GABAergic and serotonergic systems are both implicated in the modulation of neural signaling within the SCN circadian pacemaker, these systems generally work in concert in the circadian system (Rosenwasser, 2003), so the opposing association of serotonergic and GABAergic tone in genetic ethanol preference is difficult to relate to the present data. In addition, P rats show reduced expression of neuropeptide Y (Clark et al., 1998; Thiele & Badia-Elder, 2003), another known modulator of SCN signaling (Rosenwasser, 2003). Unfortunately, there are apparently no data currently available on the expression of these neuromodulators, or

their receptors, within the SCN or other neural components of the circadian system in ethanol-preferring and non-preferring rats.

These results contribute to a growing literature on the chronobiology of ethanol and ethanol preference, and are complementary to previous work in this and other laboratories showing that both adult and perinatal ethanol treatments alter circadian activity rhythms and circadian pacemaker function in unselected rats and hamsters (Dwyer & Rosenwasser, 1998; Farnell et al., 2004; Joy & Turek, 1989; Mistlberger & Nadeau, 1992; Rosenwasser et al., 2005a,b; Sei et al., 2003). In addition, similar ethanol treatments also alter the expression of neuropeptides (Clark et al., 1998; Madeira et al., 1997; Madeira & Paula-Barbosa, 1999; Thiele & Badia-Elder, 2003), growth factors (Allen et al., 2004), and circadian clock genes (Chen et al., 2004) and promote glial proliferation (Satriotomo et al., 1999) in the SCN. In addition, mutation of the circadian clock gene, *Per2*, results in increased ethanol intake in mice, indicating that this gene participates in the expression of both circadian pacemaker and ethanol preference phenotypes (Spanagel et al., 2005). It would be of considerable interest to further characterize possible interactions between ethanol preference and ethanol intake in the regulation of both behavioral and neurobiological aspects of circadian timing. Finally, more work should also be done on the possible contribution of individual chronobiological differences to the prediction of risk for alcoholism and relapse.

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