Temporal Precision in the Mammalian Circadian System: A Reliable Clock from Less Reliable Neurons

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Abstract The mammalian SCN contains a biological clock that drives remarkably precise circadian rhythms in vivo and in vitro. This study asks whether the cycle-to-cycle variability of behavioral rhythms in mice can be attributed to precision of individual circadian pacemakers within the SCN or their interactions. The authors measured the standard deviation of the cycle-to-cycle period from 7-day recordings of running wheel activity, *Period1* gene expression in cultured SCN explants, and firing rate patterns of dispersed SCN neurons. Period variability of the intact tissue and animal was lower than single neurons. The median variability of running wheel and *Period1* rhythms was less than 40 min per cycle compared to 2.1 h in firing rate rhythms of dispersed SCN neurons. The most precise SCN neuron, with a period deviation of 1.1 h, was 10 times noisier than the most accurate SCN explant (0.1 h) or mouse (0.1 h) but comparable to the least stable explant (2.1 h) and mouse (1.1 h). This variability correlated with intrinsic period in mice and SCN explants but not with single cells. Precision was unrelated to the amplitude of rhythms and did not change significantly with age up to 1 year after birth. Analysis of the serial correlation of cycle-to-cycle period revealed that approximately half of this variability is attributable to noise outside the pacemaker. These results indicate that cell-cell interactions within the SCN reduce pacemaker noise to determine the precision of circadian rhythms in the tissue and in behavior.

Key words SCN, pacemaker, oscillator, stability, Period gene, mPer1, multielectrode

Circadian rhythms in a variety of behaviors have been noted for their high temporal precision (DeCoursey, 1960; Enright, 1980). The daily onset of running-wheel activity in rodents, for example, has a standard deviation of less than 2% of the average period in the absence of environmental timing cues (Pittendrigh and Daan, 1976; Welsh et al., 1986; Daan and Oklejewicz, 2003). While the identification of key clock genes has produced a detailed model for the intracellular generation of near 24-h rhythms (Reppert and Weaver, 2002), little is known about the biological basis for the remarkable accuracy of the circadian clock.

In mammals, the SCN of the hypothalamus drives daily rhythms in locomotor activity (Klein et al., 1991). Precision of circadian behavior in rodents and humans may depend on age, sex, free-running period, the level of activity, and the duration of daily activity. Period instability may also be related to fluctuations in pacemaker properties (period, waveform, and ampli-

1. To whom all correspondence should be addressed: Erik D. Herzog, Box 1137, Department of Biology, Washington University, St. Louis, MO 63130-4899; phone: 314-935-8635; fax: 314-935-4432; e-mail: Herzog@biology.wustl.edu.

JOURNAL OF BIOLOGICAL RHYTHMS, Vol. 19 No. 1, February 2004 35-46
DOI: 10.1177/0748730403260776
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tude) and processes downstream of the pacemaker (Aschoff et al., 1971; Pittendrigh and Daan, 1976; Fitzgerald and Zucker, 1976; Penev et al., 1997; Scarbrough et al., 1997; Daan and Oklejewicz, 2003). Although little is known about the source of circadian precision, computational models have predicted that coupling between nonlinear oscillators can synchronize and stabilize the periods of the component oscillators (Winfree, 1967; Enright, 1980; Strogatz and Mirollo, 1988). Compelling evidence indicates that the SCN is a multioscillator structure, composed of cells that can oscillate independently when grown at low density in vitro (reviewed in Shirakawa et al., 2001). These cells normally synchronize to each other to coordinate rhythms, including firing rate, vasopressin secretion, and *Period1* (*Per1*) gene expression (Green and Gillette, 1982; Earnest and Sladek, 1986; Herzog et al., 1997; Yamazaki et al., 2000). To better understand where precision is determined in the circadian system, we measured the day-to-day variation of rhythmicity in cells, tissues, and behaviors of mice. We found that precision appears to be determined by cell-cell interactions within the SCN.

MATERIALS AND METHOD

Animals

Transgenic mice expressing the firefly luciferase gene under the control of the *Per1* promoter (*Per1-luc*) were generated using methods similar to those previously published (Yamazaki et al., 2000). *Per1* is one of at least 8 genes that have been implicated in regulation of circadian rhythmicity (Reppert and Weaver, 2002). A 6.7-kb genomic fragment of the mouse *Per1* gene was ligated directly to the second codon of the firefly luciferase cDNA flanked by the SV40 late polyadenylation signal. The *Per1* fragment includes five functional E box regions; a transcription initiation site; the first and second exons, which are split by the first intron; and a translational start codon in the second exon. The linearized reporter fragment was microinjected into 63 cryopreserved zygotes of C57Bl/6J (Nippon Crea, Japan) mice (Nakao et al., 1998). Transgenic mice were identified by polymerase chain reaction, and the copy number of the transgene was determined by Southern analysis. Two transgenic mice were obtained by the screening of 8 weaned pups. Transgenic mice were crossed with C57Bl/6J mice. Both founder mice developed normally. A transgenic line (Lump 1-1-8), which showed circadian oscillation of luciferase activity in the SCN, was selected for further study. There are approximately 15 copies per genome of the transgene integrated in this line. Homozygous offspring were maintained in the Hilltop animal facility at Washington University and used for behavioral and bioluminescence recording. All procedures were approved by the Animal Care and Use Committee and conformed to National Institutes of Health guidelines.

Locomotor Behavior

Thirteen male mice (21–359 days of age, from 7 different litters) were individually housed in cages outfitted with a running wheel in ventilated chambers illuminated by fluorescent bulbs (F30T12-SP41-RS, General Electric, USA; 4×10^{17} to 7×10^{18} photons/s/cm^2 at the bottom of the cages). Wheel-running activity was recorded in 1-min bins (Clocklab, Actimetrics, Evanston, IL) for 3 days in a light-dark (LD) schedule (on at 7:00 a.m. and off at 7:00 p.m.) and then for 22 days in constant darkness (DD). Animals were given fresh water and food weekly and a clean cage after 11 days in DD.

Per1 Gene Expression

SCN were harvested from 10 of the mice used in the behavioral assays. *Per1* gene activity was measured using methods similar to previous reports (Yamazaki et al., 2000; Wilsbacher et al., 2002; Abe et al., 2002). Briefly, mice were anesthetized under dim light by CO_2 inhalation, rapidly decapitated, and enucleated, and their brains were transferred to cold Hanks Buffered Saline (Sigma, St. Louis, MO). Brains were sectioned coronally with a vibratome, and 300-µm sections containing the SCN were identified under a dissecting microscope. The SCN were explanted with scalpels and placed on membrane inserts (Millicell-CM, Millipore, Bedford, MA) with 1 ml of culture medium (pH 7.2). Medium consisted of DMEM (Sigma) supplemented with 10 mM Hepes (Sigma), 2% B27, 25 U/ml Penicillin, 25 µg/ml Streptomycin, 2.2 mg/ml $NaHCO_3$, 4 mM L-Glutamine, and 0.1 mM beetle luciferin (Promega, Madison, WI). Unless noted, medium ingredients were purchased from

Invitrogen (Carlsbad, CA). Each culture was sealed in a Petri dish and maintained at 36 °C in darkness, and its bioluminescence was collected in counts per minute for 15 days with a photomultiplier tube (HC135-11MOD, Hamamatsu Corp., Shizouka, Japan). We analyzed results from cultures of bilateral ($n = 8$), isolated left ($n = 3$), and right SCN ($n = 2$). Data were omitted from 1 right SCN because it did not oscillate significantly for the criterion 7 days.

Multielectrode Recordings

Long-term firing rate patterns were recorded from cells dispersed from the SCN of C57Bl/6 mice. Founders were purchased from Harlan (Indianapolis, IN) and bred in the Hilltop animal facility. The SCN from 4 pups (postnatal age 3–5 days) were pooled, dispersed, and plated at approximately 3000 cells/mm^2 onto multielectrode arrays (Applied Neuronal Network Dynamics, Inc., Dallas, Texas) according to published methods (Herzog et al., 1998). Cells were cultured at 36 °C in 5% CO_2/95% air for 6 to 26 days in DMEM (pH 7.2; Sigma) supplemented with 2% B27, 25U/ml Penicillin, 25 µg/ml Streptomycin, 2.2 mg/ml NaHCO3, 4 mM L-Glutamine, and 10% neonatal calf serum. Half of the medium was exchanged approximately every 2.5 days with medium lacking serum, and every 1.5 weeks, cultures were treated for 24 h with 10 µM cytosine arabinoside (Sigma) to reduce glial proliferation. After 6 to 26 days in vitro, cultures were transferred to a closed recording chamber with 1 ml of recording medium (identical to that used in *Per1-luc* recording but without luciferin). We simultaneously recorded extracellular voltage signals from 2 cultures maintained on a heated stage at 36 °C in air. Signals from up to 8 electrodes were simultaneously amplified with an overall gain of 10,000 to 20,000 and bandpass filtered at 300 to 3000 Hz (Cyberamp 380 and 401, Axon Instruments, Union City, CA). The signals were displayed on oscilloscopes and digitized (12-bit resolution, 15 kHz minimum sample rate) on a personal computer using custom software. Every 10 minutes, the number of spikes with identical amplitude and duration was totaled and stored. Using offline analysis methods similar to published methods (Meister et al., 1994), we assigned action potentials of similar amplitude and duration to individual cells and counted impulse frequency. The shape of the impulse and presence of a clear refractory period following the impulse were used as criteria for single-unit activity. We analyzed results from 121 neurons, 85 (70%) of which expressed circadian rhythms for at least 4 days. Here, we included only results from the 23 neurons (in 8 cultures) that were rhythmic for the criterion 6 consecutive days (13.5 ± 7.5 days, mean ± SD; range of 6–21 days).

Data Analysis

To accurately measure the precision of rhythms, cycle-to-cycle period was measured for 7 days of recording from each animal, explant, or neuron using 8 phase markers. This produced 5 to 6 sequential period estimates for each marker from all records. We limited the analysis to this length to avoid gradual changes in the average period as described previously (Pittendrigh and Daan, 1976; Daan and Oklejewicz, 2003). Results were similar when we analyzed 14-day records of locomotor and explant data (data not shown). Clocklab was used to determine the onset, offset, acrophase, bathyphase, maximum phase, and minimum phase for each circadian cycle. Custom software was used to find the rising and peak phases of each cycle.

Briefly, onset and offset times were estimated by converting the data above the 20th percentile activity level to a series of 1s and the data below this threshold to a series of –1s. This thresholded activity was convolved with a template (6 h on followed by 6 h off for locomotor and *Per1-luc* records, 6 h on/10 h off for firing rate). The maxima of this convolution defined the onset and offset for each cycle of the actogram. Acrophase and bathyphase were calculated by the peak and trough of a sine function (period 20 to 28 h) fit to each day's activity. The maximum and minimum phases were defined by the actual peak or trough for each cycle of recording. Rising and peak phases were determined with "cross-over analysis" as previously described (Abe et al., 2002). Briefly, a 24-h running average was subtracted from the raw data to reduce trends in the baseline that occur over subsequent days. A 3-h running average and a 24-h running average were calculated from the detrended data set. The rising phase and peak between crossings of these two smoothed lines provided two phase markers for each cycle.

Period of *Per1-luc*, electrical, and behavioral rhythms was also estimated using chi-square

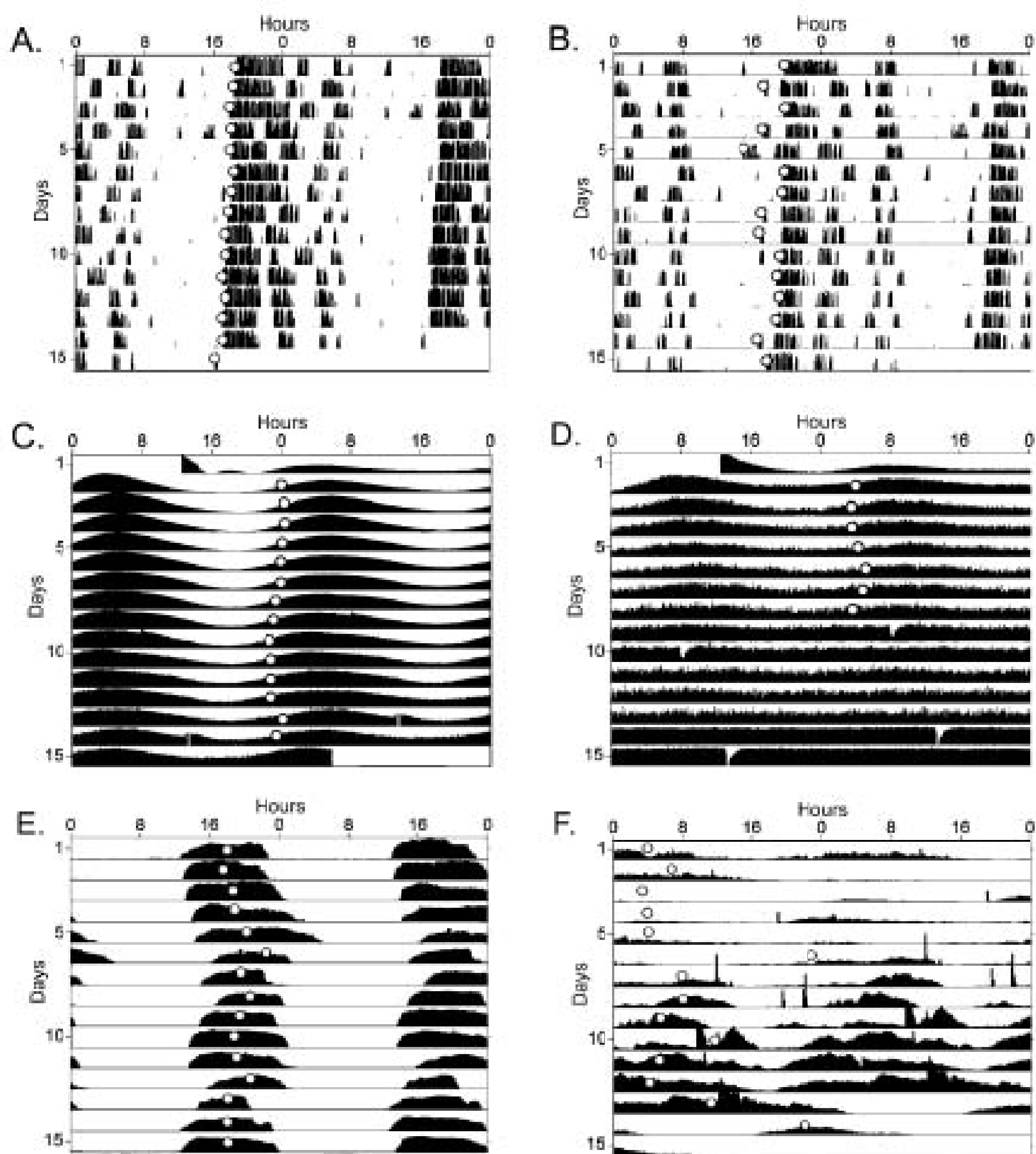

Figure 1. Determination of cycle-to-cycle stability of circadian patterns of running wheel activity (A and B), *Per1-luc* bioluminescence from cultured SCN explants (C and D), and spontaneous firing rate of single SCN neurons plated at low density (E and F). Cycle-to-cycle variation (defined as the standard deviation of 6 consecutive periods) was low in the examples on the left and higher in the actograms on the right. Each double-plotted actogram shows the rhythm recorded over 15 days, with 48 h of data on each line and the second 24 h of data replotted to the left on the line below. The onsets of locomotor activity, rising phase of *Per1-luc* bioluminescence, and acrophase of discharge rate were used as phase markers (open circles). The y axis of each line of each actogram shows 0 to 90 revolutions/min (A and B), 0 to 1 normalized bioluminescence counts (C and D), or 0 to 4 Hz (E and F). Note that phase could not be determined after 8 days of recording in D because of the loss of rhythmicity. The gaps in the data of plots C and D resulted from data collection errors.

periodogram analysis (Clocklab software) at the 0.05 significance level (Sokolove and Bushell, 1978). The amplitude of locomotor, *Per1-luc*, and firing rate rhythms was defined by the peak-to-trough height of

Table 1. Average Circadian Period and Median of the SD of the Cycle-to-Cycle Period across Animals, Explants, and Neurons

	Animals			*Explants*			*Neurons*		
	Period (h)	*SD (h)*	n	*Period (h)*	*SD (h)*	n	*Period (h)*	*SD (h)*	n
Onset	**23.88**	**0.42**	**13**	23.81	0.92	12	23.42	4.10	21
Offset	23.91	0.91	13	24.71	1.50	12	22.99	4.49	19
Acrophase	23.80	0.50	13	24.10	0.82	12	**23.50**	**2.07**	**23**
Bathyphase	23.86	0.45	13	24.01	0.72	12	23.31	2.21	19
Maximum	23.64	2.10	13	23.74	1.80	12	24.20	4.08	20
Minimum	NA	NA	0	24.14	2.10	12	23.53	4.40	21
Peak	NA	NA	0	24.19	1.18	13	NA	NA	0
Rise	NA	NA	0	**23.98**	**0.66**	**13**	NA	NA	0
Chi-square	23.90		13	24.03		13	23.89		23

NOTE: Eight different phase markers and chi-square periodogram analysis were used to estimate the average period from the indicated number of records (*n*). For each condition, numbers in bold show the phase marker that produced the lowest standard deviation, an estimate of rhythm precision. NA indicates that the data were not analyzable by the method.

the waveform averaged over 5 cycles. Statistical comparisons were made in Origin (OriginLab Corp., Northampton, MA).

RESULTS

Comparison of Circadian Phase Markers

Long-term recordings show that individual SCN neurons, SCN explants, and mice are relatively precise in their daily activity patterns (Fig. 1). Stability of these rhythms was assessed by analyzing 1 week of data from 13 animals, 13 explants, and 23 neurons using 8 different phase markers. Table 1 shows that the different markers produce average period estimates within 5% of each other. However, the standard deviation of the cycle-to-cycle period ("cycle variation") depended on the phase marker used and the type of rhythm recorded. For example, the onset of daily wheel-running activity was reliably detected for all animals and produced the lowest cycle variance. In contrast, the offset of activity was approximately twice as variable, and the cross-over and minimum algorithms failed to find reasonable phase markers due to the multiple bouts of inactivity each day. The rising phase of *Per1*-driven bioluminescence was reliably detected in all records and produced the lowest cycle-to-cycle variability. Other algorithms produced similar but slightly noisier results. The acrophase of firing rate records was reliably detected for all neurons and produced the lowest estimate of cycle-to-cycle variability. As with locomotor records, cross-over analysis failed to produce meaningful phase estimates of single-cell data. Based on these results, we used the onset of daily locomotion, the rising phase of *Per1-luc* activity, and the acrophase of firing rate rhythms to estimate the precision of the circadian system at different levels of cellular organization.

Circadian Period Varies More between Individual SCN Neurons Than It Does between Mice or SCN Explants

The mean circadian periods observed in behavior, explants, and neurons did not differ significantly from one another ($p = 0.25$, $F = 1.42$, one-way ANOVA). The variance of the period distributions, however, did depend on the conditions ($p = 0.003$, $F = 6.82$; Levene's test for equal variance). Although the variance of the periods expressed by SCN explants was almost identical to that found for behavioral rhythms ($p > 0.05$, $t = 0.13$), single SCN neurons showed a significantly broader distribution of periods than did explants or individual animals ($p < 0.05$, $t = 2.54$, and $p < 0.05$, $t = 2.67$, respectively; Bonferroni's pairwise post hoc test; Fig. 2 A-C). Even within the same culture, SCN neurons expressed periods that differed by as much as 2.63 h (0.83 ± 0.77 h, mean period difference between pairs of neurons ± SD, $n = 22$ cells in 7 cultures). This divergence of periods in the same culture has been seen in low-density dispersals from rat, hamster, and mouse SCN and provides the best evidence to date that individual SCN neurons can oscillate independently (Welsh et al., 1995; Liu et al., 1997; Honma et al., 1998; Herzog et al., 1998; Nakamura et al., 2001). Taken together, these results indicate that cell-cell interactions within the SCN synchronize SCN cells to each other and narrow the range of free-running periods expressed behaviorally.

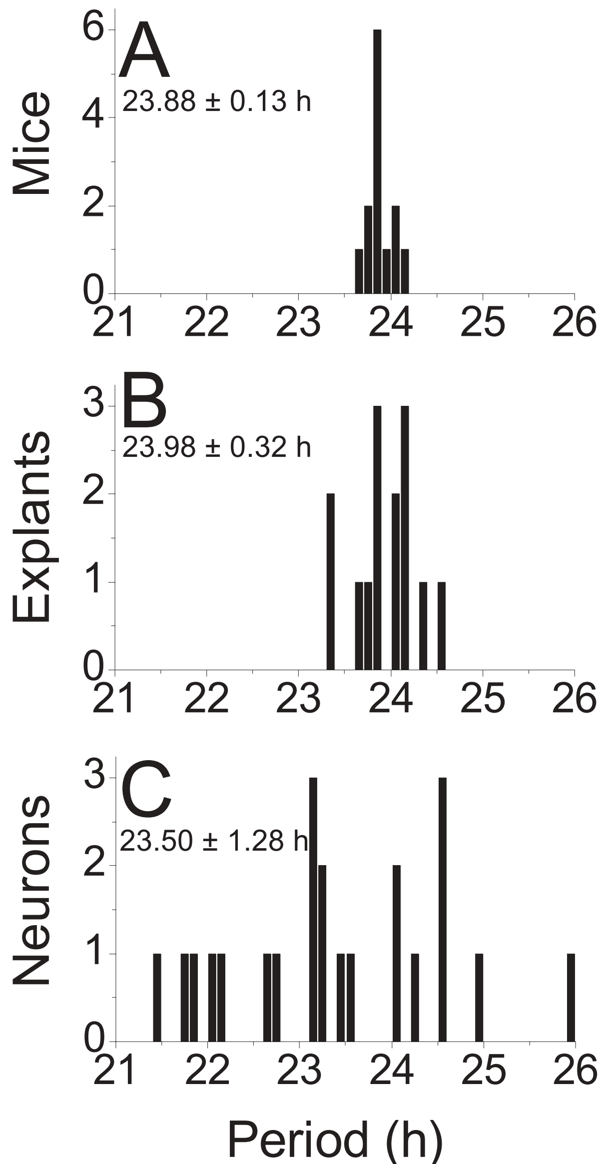

Figure 2. Circadian periods expressed by individual mice (A), SCN explants (B), and dispersed SCN neurons (C). The mean period is similar for cells, slices, and mice, but the range of periods expressed by single cells is broader than in the organized tissue or behavior. The mean period and standard deviation are given for each condition.

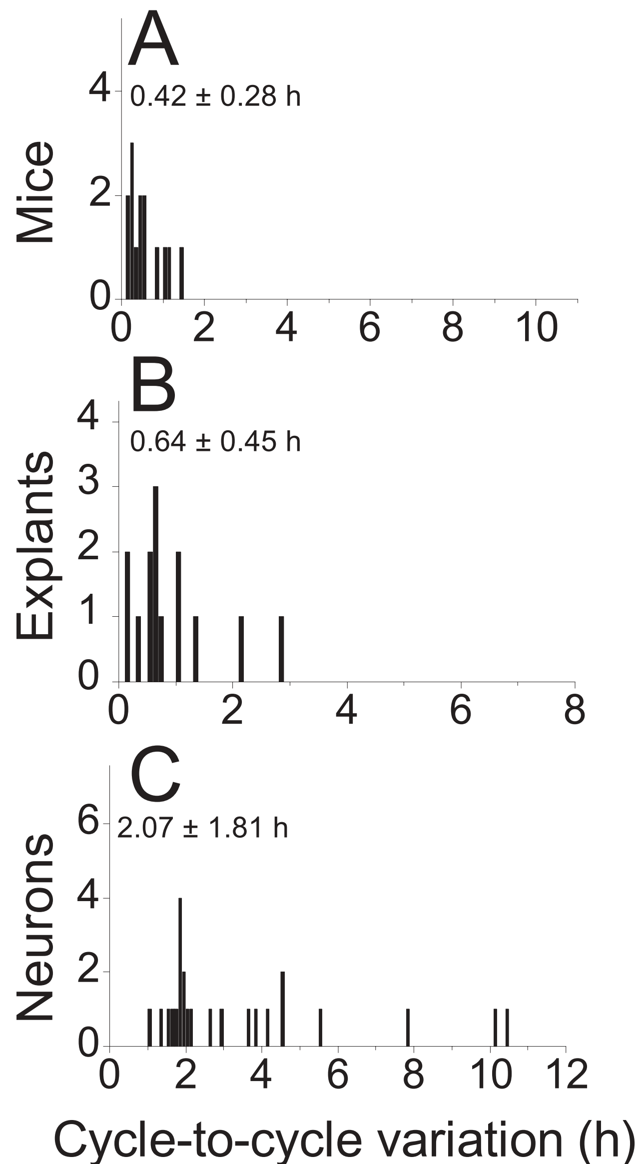

Figure 3. Cycle variation is higher in dispersed SCN neurons (C) compared to individual mice (A) and SCN explants (B). The median cycle-to-cycle variations ± first quartile are given for each condition.

Circadian Precision Was Lower in SCN Neurons Than in Explants or Behavior

Rhythms expressed by the majority of dispersed SCN neurons were less stable than those recorded from SCN tissues or mice (Fig. 3). Cycle-to-cycle variation was similar in behavior and SCN explants ($p > 0.05$) but significantly higher in dispersed SCN neurons than in behavior ($p < 0.001$) or explants ($p < 0.001$, Kruskal-Wallis test followed by Dunn's post hoc). The distributions of cycle variation were skewed toward lower values, so it is best to compare median, not mean, cycle-to-cycle variation. Dividing the median cycle variation by the average period gives a normalized estimate of cycle-to-cycle instability: 1.8% in wheel running, 2.7% in the isolated SCN tissue, and 8.8% in individual SCN neurons. Thus, mechanisms within the SCN tissue appear to reduce the noise in the circadian oscillations of SCN neurons.

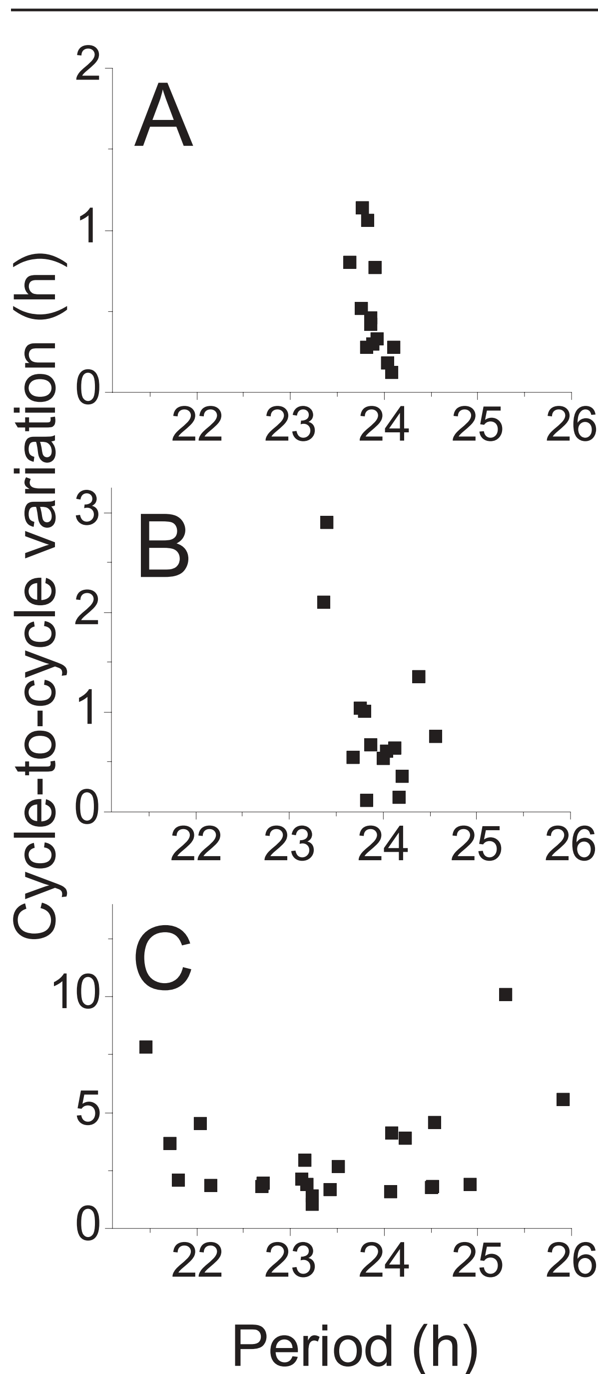

Figure 4. Cycle variation decreases as the period of mice (A) and SCN explants (B) approaches 24 h. There was no correlation between period and precision in dispersed SCN neurons (C).

Circadian Precision Correlates with Period in Behavior and SCN Explants but Not Individual Neurons

Previous work has shown that period stability tends to be higher for species and individuals with an intrinsic period closer to 24 h (Pittendrigh and Daan, 1976). Mice tend to free run in constant darkness with a period less than 24 h (Fig. 4). Even over the narrow range of periods expressed by the mice in this study, there was a strong negative correlation between period and period stability of locomotor activity so that the onsets of daily locomotor bouts were sloppier in mice with shorter periods ($r = -0.64$, $p < 0.02$). Explants also showed this correlation ($r = -0.56$, $p < 0.04$); however, individual neurons did not ($r = 0.39$, $p > 0.06$).

When we compared the performance of individual mice and their explanted SCN, we found no correlation in their periods ($p = 0.33$), their cycle-to-cycle variation ($p = 0.85$), or the amplitude of their rhythmicity ($p = 0.42$). This could indicate that the SCN does not uniquely dictate these parameters of locomotor rhythmicity or, perhaps more likely, that small differences between isogenic animals in period, stability, and amplitude of running-wheel rhythms cannot be resolved at the level of the cultured SCN.

Circadian Precision Does Not Depend on Age

Period stability has been reported to begin to deteriorate in rodents around 1 year of age (cf. Scarbrough et al., 1997; Aujard et al., 2001). We found that mice age 21 to 94 days old ($n = 9$) showed similar behavioral period stability (0.56 ± 0.12 h, mean ± SEM) to mice age 220 to 359 days old (0.58 ± 0.29 h; $n = 4$, $p = 0.94$, $t = 0.08$, Student's t test). Furthermore, age had no significant effect on the precision of rhythmicity in SCN explants (1.0 ± 0.32 h, $n = 9$ younger; 0.61 ± 0.22 h, $n = 4$ older; $p = 0.52$, $t = 0.66$). Although the period of behavioral rhythms positively correlated with age ($r = 0.57$, $p = 0.04$), the average period of young and old animals did not significantly differ (23.84 ± 0.04 h for 21–94 days old; 23.98 ± 0.06 h for 220–359 days old; $p = 0.09$, $t = 1.89$). There was no correlation between age and the amplitude of wheel running ($p = 0.81$) or explant rhythms ($p = 0.93$). These results indicate that circadian rhythms of the SCN and locomotor activity change little in their precision, period, and amplitude from weaning to at least 1 year of age.

Circadian Precision of Behavior and SCN Physiology Are Independent of the Level of Activity and the Amplitude of Rhythmicity

Aschoff et al. (1971) showed that the stability of a rhythm could theoretically depend on the waveform of the underlying pacemaker's oscillation. We found

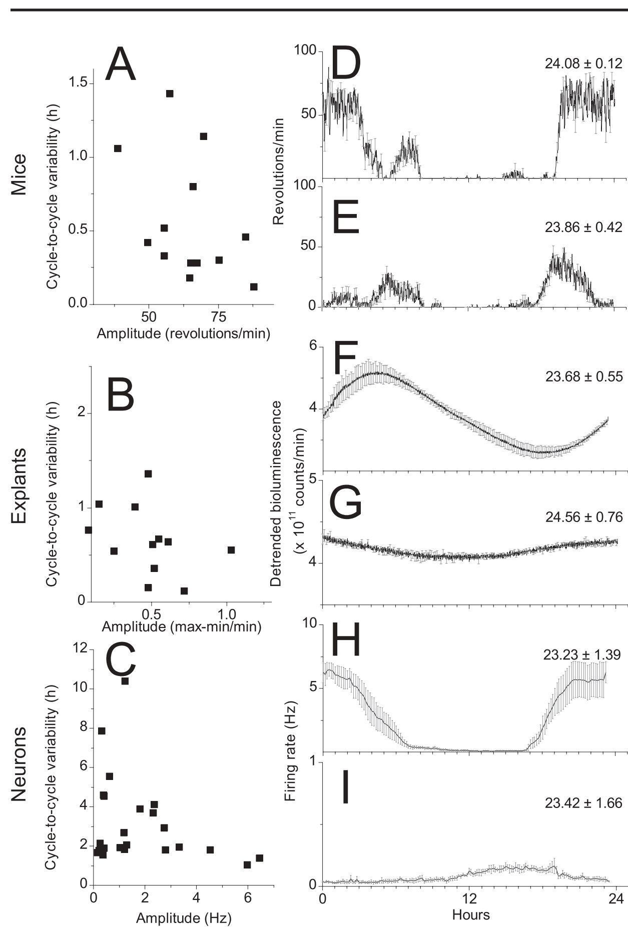

Figure 5. The peak-to-trough amplitude of rhythms in locomotor activity (A), ensemble bioluminescence of SCN explants (B), and discharge rate of single SCN neurons (C) is not a good predictor of rhythm stability. Examples of high- and low-amplitude rhythms in wheel-running behavior (D and E), *Per1* expression in SCN explants (F and G), and spontaneous firing of SCN neurons (H and I) show the diversity of circadian waveforms at each level of organization. Each profile shows the average activity from 6 days plotted over 1 circadian cycle. For clarity, error bars (SEM) are plotted every 10 minutes. The period and cycle-to-cycle variations (mean ± SD) are given for each individual.

that the average activity per cycle did not correlate with precision of behavioral ($p = 0.18$), tissue level ($p = 0.83$), or single-cell rhythms ($p = 0.42$). The amplitude of rhythmicity also did not correlate with cycle-to-cycle variation of locomotor ($p = 0.17$), explant ($p = 0.24$), or cellular rhythms ($p = 0.15$) (Fig. 5). This was especially apparent for some single neurons that had low-amplitude discharge patterns but were among the most accurate from cycle to cycle. These results indicate that neither activity level nor rhythm amplitude is a good predictor of circadian precision.

DISCUSSION

Intercellular Communication Improves the Precision of the SCN

We find that behavioral and tissue-level rhythms show increased precision as period approaches 24 h, but single SCN neurons do not. In addition, isolation of the SCN in vitro has little consequence on its period stability. This suggests that the SCN largely determines the precision of locomotor rhythmicity. However, this does not mean the SCN determines all circadian properties of all circadian rhythms, particularly given the recent discoveries of extra-SCN oscillators (reviewed in Herzog and Tosini, 2001). In addition, disrupting the normal connectivity between SCN neurons drastically reduces their period stability, so that only 2 of 23 neurons showed precision equal to or less than the 3 noisiest SCN explants or mice. Thus, it is likely that interaction between SCN cells critically improves their circadian precision.

The role of coupling circadian oscillators to reduce cycle-to-cycle noise has also been implicated in the snail eye and avian pineal where removing cells reduces the accuracy of those that remain (Block and McMahon, 1984; Robertson and Takahashi, 1988). However, current models for the intracellular generation of circadian rhythms have either assumed a noise-free set of reactions (Scheper et al., 1999; Leloup and Goldbeter, 2003) or found the resultant rhythmicity to be relatively resistant to stochastic fluctuations (Barkai and Leibler, 2000; Gonze et al., 2002; Vilar et al., 2002). The present results indicate a need to incorporate the role of cell-cell interactions in shaping circadian rhythmicity in single cells.

An Estimation of Pacemaker Precision versus Wake-up Time Precision

Pittendrigh and Daan (1976) elegantly argued that variability observed in locomotor rhythms is likely due to noise both in the pacemaker and in processes controlled by the pacemaker (termed "wake-up noise"). Assuming independence between the two sources of noise, they found that variability due to the pacemaker, $s(\tau)$, and to the wake-up processes, $s(w)$, can be estimated by $s(\tau) = \sigma\sqrt{1+2r_s}$ and $s(w) = \sigma\sqrt{r_s}$, where r_s is the serial correlation coefficient of successive periods and σ is the standard deviation of those periods. A negative serial correlation reflects the like-

lihood that a long cycle will be followed by a short cycle, a necessity for a pacemaking system that reliably oscillates while controlling a noisy process. We calculated from 7-day records of each mouse, explant, and neuron. We found the mean r_s was −0.21 for behavior, −0.18 for SCN tissue, and −0.43 for individual SCN neurons. Given our estimates of σ (SD in Table 1), we can calculate the variability due to the wake-up and pacemaking processes for mice (0.19 and 0.32 h), slices (0.27 and 0.52 h), and neurons (1.36 and 0.76 h). Thus, imprecision, or "jitter," attributable to the pacemaker is between 1.3% and 3.2% of the average cycle length in mice and cultured SCN neurons. Interestingly, noise is estimated at 1% in the wake-up processes driving behavioral and explant rhythms but 5.8% in firing rate patterns of single SCN neurons. This relative increase in wake-up noise for neurons could indicate that intercellular communication within the SCN is used to stabilize processes controlled by the pacemaker. For example, synaptic input may enhance clock control of membrane potential to stabilize rhythms in firing rate in the intact SCN. In sum, median cycle-to-cycle variation is reduced from approximately 2 h in the firing rhythms of single cells to less than 40 min in the intact SCN and mouse by mechanisms that reduce both pacemaker and wake-up noise.

Evidence That Coupling among a Small Number of Pacemakers May Determine the Period and Precision of Behavioral Circadian Rhythms

Theoretically, synchronization might discipline oscillators to be more precise (Enright, 1980; Winfree, 1980). This "collective enhancement of precision" would depend on the nature of the coupling between the oscillators. Liu et al. (1997) first proposed that coupling within the SCN could synchronize oscillators to the mean period of the population. That is, coupling might average the frequencies across oscillators. The evidence for averaging by coupling within the SCN is indirect but supported by several findings. Dispersed SCN neurons express a broader range of periods than is seen in behavior or SCN explants, whether measured from the ensemble of cells by *Per1*-driven bioluminescence or from the discharge rate of individual neurons. However, the mean periods across SCN neurons and slices in vitro match the mean behavioral period (Liu et al., 1997; Honma et al., 1998; Herzog et al., 1998; Nakamura et al., 2001). Although we do not know if or how averaging of neuronal periods occurs in the SCN, such a mechanism could have a predictable effect on the precision of individual oscillators. For example, oscillators that synchronize to the mean period can reduce their noise by approximately $\frac{1}{\sqrt{N}}$, where N is the number of symmetrically coupled, limit-cycle oscillators with identical amplitude and odd coupling (Needleman et al., 2001). Odd coupling indicates that oscillators have opposing effects on each other's frequency. In this specific case, coupling 100 oscillators, each with a cycle variation of 2 h, would result in a synchronized system with jitter of $\frac{2}{\sqrt{100}}$ or 0.2 h. Given our measured cycle-to-cycle variation of individual SCN neurons (2.1 h), we can estimate the minimum number of pacemakers needed to reduce the measured jitter to that measured for the intact system (0.42 h): 25 pacemakers. This prediction is several orders of magnitude lower than estimates based on the percentage of independently oscillating SCN neurons in dispersals (50–75%) (Welsh et al., 1995; Herzog et al., 1998) or the percentage of the SCN required to sustain rhythms in locomotor activity (at least 25%) (Rusak, 1977; Davis and Gorski, 1988; Harrington et al., 1993). It is intriguing, however, that small lesions specific to the central SCN are sufficient to abolish behavioral rhythmicity (LeSauter and Silver, 1999). This may indicate that the number of pacemakers required for SCN rhythms is considerably lower than previously thought. Alternatively, the specific assumptions used in the published models of averaging by coupling or improved precision by averaging may not apply to the SCN. Critically, it is not clear that $\frac{1}{\sqrt{N}}$ improvement results under all conditions that average the periods of the component oscillators. It will be helpful to have empirical measures of the result of coupling on the period of SCN oscillators and the nature of coupling between SCN cells. Because each SCN is composed of a heterogeneous population of approximately 8000 cells, it remains an important goal to identify the cell classes involved in rhythm generation.

Reduced Precision of Single Neurons In Vitro Is Unlikely to Be an Artifact

Do dispersed neurons show greater period instability because their health is compromised? In vitro stud-

ies always run the risk of revealing properties that do not exist in vivo. Although the inability of cells to tolerate dispersal is a reasonable explanation for greater instability, a number of studies have argued that rhythms seen in culture reflect properties intrinsic to SCN neurons (Gillette and Prosser, 1988; Zlomanczuk et al., 1991; Satinoff et al., 1993; Meijer et al., 1997; Liu et al., 1997; Herzog et al., 1998; Mrugala et al., 2000; Aujard et al., 2001; Nakamura et al., 2002; Albus et al., 2002). Here, dispersed neurons were recorded after 3 weeks in vitro so that cells injured during plating had time to recover or die. Furthermore, because explants and dispersals were recorded in the same media, it is unlikely that the difference in precision arises from culture conditions.

A more fundamental concern is that it may be inappropriate to infer the precision of the pacemaker from observed firing rate rhythms, as these rhythms may reflect pacemaker output rather than the clock itself. *Per1* expression or other intracellular events might have more stable periods in isolated cells than firing rate. Previous work, however, has indicated that membrane excitability is likely to be closely related to pacemaker state under most conditions (McMahon and Block, 1987; Nitabach et al., 2002; Pennartz et al., 2002; Panda et al., 2002; Kuhlman et al., 2003). In addition, our estimates of pacemaker variability, attempting to account for noise in nonpacemaker events, still found the jitter of single neurons to be more than double that of SCN explants.

Taken together, these results indicate the precision of the circadian system derives from the interactions of multiple, sloppier pacemakers within the SCN. These stabilizing interactions between individual SCN neurons are qualitatively similar across mice of varying ages and activity levels and appear to be stronger in animals with behavioral periods closer to 24 h.

ACKNOWLEDGMENTS

The title of this article was inspired by the title of J. T. Enright's (1980) article in *Science*. We are grateful to Dr. Martin Straume for developing some of the analytical tools used in this article and to Obinna Ndum, Daniel Granados-Fuentes, Ute Abraham, and Ed Spitznagel for helpful discussions. This work was supported by National Institutes of Health grant MH63104, the McDonnell Foundation for Higher Brain Function (EDH), and National Science Foundation Graduate Student Fellowship (SJA).

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