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Interval timing in mice does not rely upon the circadian pacemaker

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Abstract

The suprachiasmatic nucleus (SCN) of the hypothalamus is a precise timekeeper that controls and synchronizes the circadian period of countless physiological and behavioural functions and entrains them to the 24 h light/dark cycle. We examined the possibility that it is also indirectly involved in measurement of a briefer interval by observing the effects of lesions targeted at the SCN, and abolishing circadian rhythmicity, upon interval timing behaviour. Fourteen house mice (*Mus musculus*) were trained to estimate a 10 s interval using a modified peak procedure, and then underwent electrolytic lesions. Six individuals became behaviourally arrhythmic. Peak interval performance was then assessed in 12:12 light/dark conditions and in constant darkness. No significant change in peak characteristics was observed as a consequence of the lesion for either rhythmic or arrhythmic groups. These results show that the accurate measurement of 10 s requires neither a functioning circadian pacemaker nor entrained behavioural rhythmicity.

Keywords: Circadian rhythms; Peak procedure; Interval timing; Suprachiasmatic nucleus; Operant conditioning; Time perception

The circadian clock provides a precise timer of the endogenous circa 24 h period, and is kept in synchrony with the external world through light–dark (LD) entrainment [6]. Since the scale and genetically pre-programmed nature of circadian timing is so different from much of behavioural timing, in which events of seconds or minutes are incorporated by learning, the two forms of timing are usually thought of as distinct phenomena. There is, however, evidence to suggest that circadian timing can interact with timing of both much shorter [1,5,8,10], and much longer [11] intervals. One fairly radical possibility is that the circadian clock may be part of an array of oscillators used directly to measure briefer intervals [3]. Alternatively, the circadian clock could synchronize or calibrate mechanisms used for interval timing [4]. In a third scenario, rather than the circadian clock itself, regular activity cycles entrained either by external cues or by the endogenous clock could be used to synchronize mechanisms for interval timing.

The circadian 'master clock' has been localized in the suprachiasmatic nucleus of the hypothalamus (SCN) [6]. Removal of the SCN eliminates circadian rhythmicity in behavioural activity patterns when animals are kept in constant darkness (DD). In LD light regimes, rhythmicity is maintained even in SCN lesioned animals as a consequence of direct or masking effects. Here, we examine the potential for a link between circadian timing and measurement of briefer intervals by exploring the effect of SCN lesions upon estimation of a 10 s duration by house-mice (*Mus musculus*). Lesioned animals were tested first under entrained LD conditions and then while free-running in DD. If the circadian oscillator is required directly for short interval timing, or to entrain other timers, lesioned animals are expected to show a timing deficit, even when entrained by LD conditions. If circadian rhythmicity is necessary, but the SCN pacemaker is not specifically required, then lesioned animals should only show a deficit when maintained in DD.

Fourteen male C57/Bl6 house-mice (*M. musculus*) aged 7 weeks and housed individually in a 12:12 LD cycle were used. Their daily ration was approximately 4 g rat chow (Hope Farms), maintaining them near 90% of their free-feeding body weight.

Two Coulbourn mouse operant boxes, each isolated in a soundproof chamber and with response lever and food hopper side by side, were used for the behavioural task. A Coulbourn Liquid Dipper, which delivered 0.3 ml portions

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of condensed milk, provided reinforcement. A dim red LED was used to light the food hopper during training. The unmodified Coulbourn mouse-response lever required a force of approximately 5 g to register a response, but a 2 cm extension of rigid plastic facilitated pressing, reducing the required force to approximately 1 g. A Coulbourn speaker mounted high on the wall was used to provide 2.9 kHz auditory cues. Data collection and control of the operant boxes were accomplished using an Acorn computer.

We trained animals to estimate a 10 s interval using a modification of the peak procedure [9]. This is similar to the fixed interval procedure [2] in which animals are conditioned to expect a reward in response to a lever press made after a fixed criterion duration. In both procedures, animals increase the frequency of lever pressing in parallel with expectation of reward as the criterion time approaches. In the peak procedure, probe trials are inserted into the sequence of trials such that the animal's estimate of the reward time can be determined using the peak in unreinforced responses, which decrease back to baseline after this point, producing something like a Gaussian function of response frequencies.

For the initial conditional association, animals experienced a condition in which any lever response earned an immediate reward, signalled by a red light in the hopper, during 15 daily sessions of 10 min each for 3 days.

For peak procedure fixed interval training, animals then performed two sessions per day in which rewards were available only during an interval 10–14 s after trial onset. Trials were marked by a high pitched tone and were randomly spaced within 20 min sessions using inter-trial intervals (ITI) chosen from a beta distribution with a mean of 3.2 s and a standard deviation of 2.1 s. Responses during the target interval were reinforced by cessation of the trial/tone and by delivery of a condensed milk reward. If no reward was earned (no lever press between 10 and 14 s), the trial continued to a total of 45 s, with an additional interval chosen from the ITI array.

For peak procedure probe trials, after approximately 2 weeks of training with the fixed interval schedule, unreinforced probes were introduced comprising 20% of the total trials. Probes lasted 45 s, plus an interval from the ITI distribution mentioned above.

All animals underwent surgery when approximately 17 weeks old. They were anesthetized with sodium pentobarbital (50 mg/kg, i.p.), treated with atropine sulphate (0.25 mg/kg, i.m.) and placed in a stereotaxic apparatus (Kopf Instruments, Tujunga, CA). The scalp was shaved and cleaned, an incision was made, and the skull bone was drilled. A monopolar macroelectrode (tungsten, TM33B10, WPI, Germany) was aimed bilaterally at the SCN (AP +0.1 mm anterior to bregma, L 0.2 mm on both sides of midline, V −5.4 from the skull). DC current (0.5 mA) was passed for 20 s. The skin was then stitched and the wound protected with antibacterial ointment (Neosporin).

Behavioural circadian rhythmicity was assessed for 10 days, beginning more than 30 days post-lesion, using continuous access to running wheels (approximately 15 cm in diameter) in constant dim red light (DD; <2 mW/m^2, λ_{max} 773 nm). Running wheel data were plotted as double-plot actograms that were used for visual assessment of rhythmicity; in Fig. 1 only single-plot actograms are shown. The χ^2 periodogram with DQ(P) statistics was computed on a 10 day interval using running wheel data [12].

Peak procedure analysis was restricted to responses prior to 10 s on reinforced trials, and up to 30 s on probe trials. Trials were pooled across multiple sessions to provide data for the 2 days immediately pre-lesion, for 2 days immediately post-lesion, for 10 days immediately post-lesion, for the last 2 days of the LD condition before DD (days 18 and 19 post-lesion), and for the 11th and 12th days of the DD condition (days 30 and 31 post-lesion). Pooled response times were partitioned into 2 s time bins and histograms were produced by plotting mean responses per second against these bins. Gaussian curves were then fitted to each histogram using an iterative least squares fitting routine written in MatLab. This routine performed a grid search with integer values for mean (1:60 s) and standard deviation (1:20 s), and with precision up to two decimal places for amplitude (0.05:1.05 responses s^{-1}). All possible combinations of parameters were tried, and the combination associated with the lowest residual values (least-squares fitting method) was selected. Two-tailed t-tests were performed to compare parameters from Gaussian curves fitted to the data before and after the lesion and before and during the free-running DD condition. Probabilities were Bonferroni corrected for multiple comparisons. To check the goodness of fit between each Gaussian curve and the data it was fitted to, a regression was performed between the histogram value in each bin and the value of the Gaussian at that time-point; probability was calculated from the resulting R statistic. For each group (rhythmic and arrhythmic), weighted mean Gaussian curves were found by inversely weighting the three parameters (mean, standard

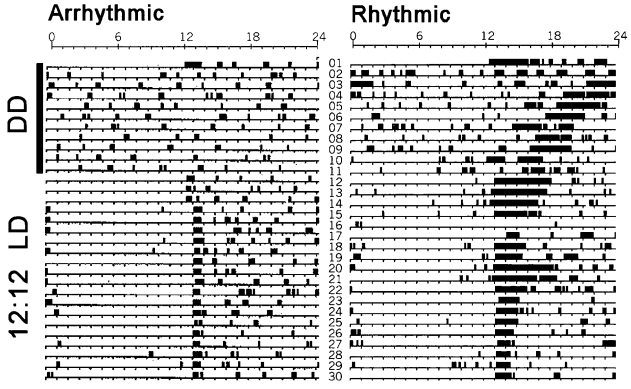

Fig. 1. Sample actograms from arrhythmic mouse A5 and rhythmic mouse R7. Time is marked in hours along the horizontal axis. Successive days are stacked on the vertical axis starting at the top. Black tick marks represent turns of the running wheel. For the first 10 days shown, the mice were kept in DD, and on day 11 they were switched to LD 12:12.

deviation, and amplitude) from each individual in the group by the sum of squares of the residuals for that individual.

Although all animals underwent the same surgery, only some displayed a loss of circadian rhythmicity as a result. Destruction of the SCN induces circadian arrhythmicity [6, 12], however in *M. musculus* this structure is extremely small and difficult to locate. It is therefore probable that our lesions successfully destroyed the SCN only in those animals which became arrhythmic. Unfortunately, histological reconstruction of these lesions is not available. Fig. 1 shows sample actograms for an arrhythmic and a rhythmic mouse in both DD and LD conditions. Table 1 lists the rhythmicity assessments for all 14 mice. Columns 2 and 5 of Table 1 show the assessments made by visual inspection of the actograms for DD and LD, respectively. Columns 3 and 6 show the DQ(P) statistical assessment. Animals were divided into two groups: those maintaining rhythmicity in DD (rhythmic group), and those not maintaining rhythmicity (arrhythmic group). All visually rhythmic mice had DQ(P) in DD > 100, while all visually arrhythmic mice had DQ(P) < 100, except for one mouse, A7, which had a period of 25.3 h. The normal circadian period in *M. musculus* is about 23 h, so the pacemaker in this animal was clearly disrupted, though it maintained periodicity. Because of this ambiguity, all statistics on peak interval data were computed both with mouse A7 in the arrhythmic category and with it excluded completely. Both resulting P values were considered.

Table 1
Rhythmicity assessment

Mouse	Constant dark (DD)			Light/dark (LD) 12:12		
	Visual	Period	DQ(P)	Visual	Period	DQ(P)
A1	A	–	95.59	R	24.00	938.84
A2	A	–	85.10	R	23.93	158.58
A3	A	–	71.55	R	23.27	140.13
A4	A	–	84.77	R	24.00	1401.37
A5	A	–	85.23	R	23.87	586.20
A6	A	–	56.64	R	23.93	394.47
A7*	A	25.33	185.23	R	23.93	800.59
R1	R	23.33	113.37	R	24.00	691.97
R2	R	23.00	224.66	R	23.67	403.58
R3	R	23.87	289.66	R	24.00	408.51
R4	R	23.60	176.94	R	23.93	959.75
R5	R	23.67	632.72	R	24.00	608.87
R6	R	23.73	137.94	R	23.93	213.50
R7	R	23.13	1134.0	R	23.93	1773.33

Results from visual and statistical assessment of mouse rhythmicity during 10 days in DD and in LD 12:12 conditions. Visual assessment is based upon the pattern of activity apparent in double-plotted actograms. Statistical assessment used the DQ(P), a measure of periodicity where DQ(P) > 100 is accepted as significant periodicity [10]. Rhythmicity was estimated from 10 days of activity counts for each condition. Mice have been divided into two groups of seven, arrhythmic (A) and rhythmic (R). Note that all animals' behaviour is 'rhythmic' in LD conditions. *Mouse A7 had a significant DQ(P) score but its wheel running pattern was visually assessed as arrhythmic, and it showed an unusually long circadian period in DD.

We did not detect any change in behavioural performance on the peak interval procedure as a consequence of surgery in either LD or DD conditions. No significant change in the mean, standard deviation or amplitude of Gaussian curves fitted to the response histograms was detected for either rhythmic or arrhythmic groups between either 2 days prior to the lesion and 2 days post-lesion (all data collected in LD conditions), or the last 2 days in LD conditions (post-lesion) and days 11 and 12 of the DD condition (see Fig. 2 for sample curves).

The Gaussian curves provided a significant fit the data ($P < 0.05$) for all but one mouse in the arrhythmic group, both before and after the lesion. The fits were also significant for all but three mice in the rhythmic group during LD conditions. Parameters of the Gaussians fitted to each condition were compared with t-tests both with and without the data from these animals, with no difference in results. Probabilities associated with curves fitted to the data from 2 days before DD and the last 2 days of DD were higher (mean $P = 0.2$), with data from only two arrhythmic and two rhythmic animals providing a significant fit both before and during DD. Hence, it was not possible to restrict our statistical analysis using only data from animals where the fit reached significance. It is worth noting that the poor curve fitting occurred in both rhythmic and arrhythmic groups, both before and during the period of DD. It cannot, therefore, be explained either as a response to the lesion or to circadian arrhythmicity, but may be due to some confounding factor such as prolonged training, or the age of the animal.

Three hypotheses were examined in this experiment. The first two concerned the primary circadian pacemaker in the SCN: is this necessary for timing of 10 s intervals, either directly, or by synchronizing timers used for that duration? Does its removal have any effect upon performance in a peak procedure task to which animals have been previously trained? In our data, hypothalamic lesions targeting the SCN, which rendered half of the animals completely arrhythmic in DD, produced no significant change in the peak position, standard deviation, or amplitude of the timing response function when this arrhythmic group was tested in LD 12:12 conditions. Hence the first two hypotheses were not supported. This result is in keeping with a study in rats which showed that SCN lesions had no effect upon performance in a fixed interval task wherein animals measured 1 min [7].

The third hypothesis we examined was whether maintenance of a circadian activity pattern is necessary for measurement of 10 s. If entrainment to the 24 h cycle is important for keeping different timing mechanisms calibrated with respect to one another, then keeping arrhythmic animals in DD where they have no access to circadian cues should lead to gradual desynchronization of these mechanisms. This should be evident as an increase in the variance associated with the estimation of a 10 s interval. We compared curves fitted to data collected while arrhythmic animals were maintained in DD to those fitted to data

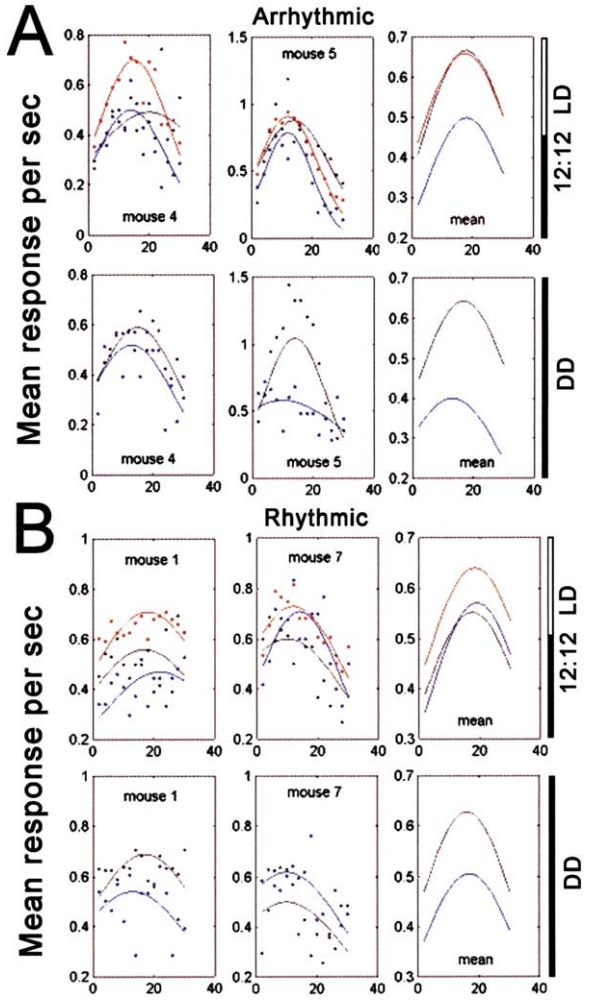

Fig. 2. Scattergrams of mean responses per second in 2 s time bins for sample arrhythmic (A) and rhythmic (B) animals. Best-fit Gaussian curves are displayed as solid lines. The top row shows data collected in LD conditions during the last 2 days immediately pre-lesion (BLACK), the first 2 days of data collection post-lesion (BLUE), and the first 10 days of data collection post-lesion (RED). The bottom row shows data collected during the last 2 days of the LD condition (BLACK) and the last 2 days of the DD condition (days 11 and 12, BLUE), all of which is post-lesion. Gaussian curves calculated using the weighted mean parameters across all animals in each group (rhythmic/arrhythmic) are also shown for each condition.

collected while they experienced an entraining 12:12 LD environment, and found no significant change in any parameter. These results argue against the third hypothesis. However, it remains possible that a more extended period of isolation from temporal cues would lead to a measurable change in the 10 s timing function. Recent evidence suggests that at a molecular level, circadian oscillations are present in other tissues such as liver, lung and heart, with some degree of phase independence from the SCN [13]. Obviously, our experiments do not exclude the possibility that such peripheral circadian oscillations play a role in short-term interval timing.

Our data demonstrate that mice in which behavioural circadian rhythmicity has been eliminated show no significant alteration in their ability to measure 10 s. The data thus show that for *M. musculus*, an intact circadian clock in the brain is not required for the accurate measurement of learnt short intervals, at least for the length used here. Our data also suggest that entrainment to a 12:12 light/dark cycle is not necessary for the interval timing function produced by either rhythmic or arrhythmic mice, thus implying that circadian rhythmicity and entrainment are not required for the accurate measurement of 10 s intervals. Overall, these data support the independence of mechanisms for circadian and interval timing.

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