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The suprachiasmatic nuclei: circadian phase-shifts induced at the time of hypothalamic slice preparation are preserved in vitro

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Neurons of the suprachiasmatic nuclei (SCN) of the hypothalamus compose a primary oscillator which organizes circadian rhythms in mammals. In cultured hypothalamic slices from rat brain, the SCN diurnal oscillation in neuronal firing rate continued unperturbed when slices were prepared during the light phase of the donor's light/dark cycle. However, when slices were prepared during the donor's dark period, the rhythm was phase-shifted. The sign and shape of the phase-response relationship for resetting in the isolated oscillator is very similar to that for intact animals, except that in isolation the SCN oscillator undergoes large shifts during the first cycle. The finding that a phase-shifting stimulus at the time of brain slice preparation causes normal phase readjustment in vitro demonstrates that the underlying mechanism is endogenous to the SCN and can be probed in the brain slice.

The suprachiasmatic nuclei (SCN) at the base of the mammalian brain orchestrate many daily oscillations of physiological, metabolic and behavioral rhythms[10,14,16,17,22,23,25,28–31]. The neurons of these nuclei exhibit a circadian oscillation in firing rate, which peaks near midday in nocturnal rodents[13]. They can be reset when light stimuli appear during the animal's subjective night so that the oscillation rises and falls at a new phase. They also continue to maintain their characteristic circadian pattern of firing rate whether semi-isolated by knife cuts in vivo[12,13] or removed from the animal and cultured in the hypothalamic slice in vitro[4,5,27]. Oscillations in the activity of adjacent hypothalamic nuclei, which control such circadian functions as locomotion, eating, drinking, sleep/wakefulness and hormone release, disintegrate under each condition where inputs from the SCN are removed[12]. Thus, the neurons of the SCN form an endogenous oscillator which transmits time information to other parts of the brain.

The discovery that the SCN oscillator survives isolation using the hypothalamic brain slice technique[6] and continues apparently unperturbed[4,5], provides compelling evidence that the neurons in the slice possess the information to keep 24 h time. However, no studies have yet focused on the mechanism which drives this neuronal oscillator in isolation. In the course of preliminary experiments aimed at examining the phase-shifting mechanism of the SCN in vitro, I found that the circadian clock is reset at the time the animal is killed and the brain slice prepared if this occurs during the dark part of the light–dark cycle. Further, the adjustment of the phase of the oscillator takes place apparently normally during the first circadian cycle in vitro.

These studies were performed on 2–5-month-old Long–Evans rats which had been bred and reared in our laboratory to reduce individual differences. Animals were housed on 12:12 h light–dark cycle. They had been handled at random intervals to reduce stress at the time of decapitation. Each rat was maintained on its entrained lighting condition until initiation of the brain slice procedure. At that moment, the donor was taken from the cage, into laboratory light-

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ing, decapitated and the brain quickly removed; thus, an animal in the dark phase of the lighting cycle experienced 20–30 s of ambient light during handling, decapitation and surgery until the optic tracts were severed.

After brain removal, the hypothalamic region was blocked and 400 or 500 μm slices of the suprachiasmatic region were prepared with a manual chopper. Slices containing the SCN were allowed to equilibrate for 1 h in a Hatton-style brain slice chamber[6] before commencing electrical recordings. Slices were continuously perfused with Earle's Balanced Salt Solution (GIBCO), supplemented with $NaHCO_3$ (26.2 mM) and glucose (19.0 mM), pH 7.2 and saturated with 95% O_2:5% CO_2. Under these conditions SCN activity can be recorded for 24–36 h.

Extracellular electrical activity of single neurons was recorded with glass microelectrodes filled with 5 M NaCl. Each electrode was slowly advanced with a Narishige MO-8 hydraulic microdrive until a unit was encountered. Signal:noise ratio of >2:1 was the criterion for adequate differentiation of a unit for counting. Units were isolated with a WPI 121 window discriminator and activity was allowed to stabilize for at least 2 min before counting. The pattern and rate of firing were analyzed by a small computer. The data thus generated from randomly encountered neurons from SCN in a single slice were plotted against circadian time of the donor (CT, where the clock starts at 'lights on' and continues for 24 h) in order to determine the peak of the next electrical oscillation. Half of the animals used to study the effect of slice preparation during the dark phase had been housed on a reversed lighting cycle for a minimum of 3 weeks; the results for a given time are completely overlapping with those from rats with lights on during the day.

The relationship of circadian time of suprachiasmatic isolation to the phase of the next oscillation in firing rate of SCN neurons in vitro was systematically examined. Brain slices were prepared from a total of 16 donor rats at a range of times in their circadian cycles: 6 donor rats were in the light phase of their circadian cycles (at CT 0.5, 4.0, 5.0, 5.5, 5.7 and 9.0) and 10 were in the dark phase (at CT 13.9, 14.2, 15.5, two at 16.0, 17.8, 18.5, 19.0, 19.5 and 20.0). In each experiment, single unit activity of neurons in the paired SCN isolated in a single hypothalamic brain slice was sampled over 12–28 h in order to define the

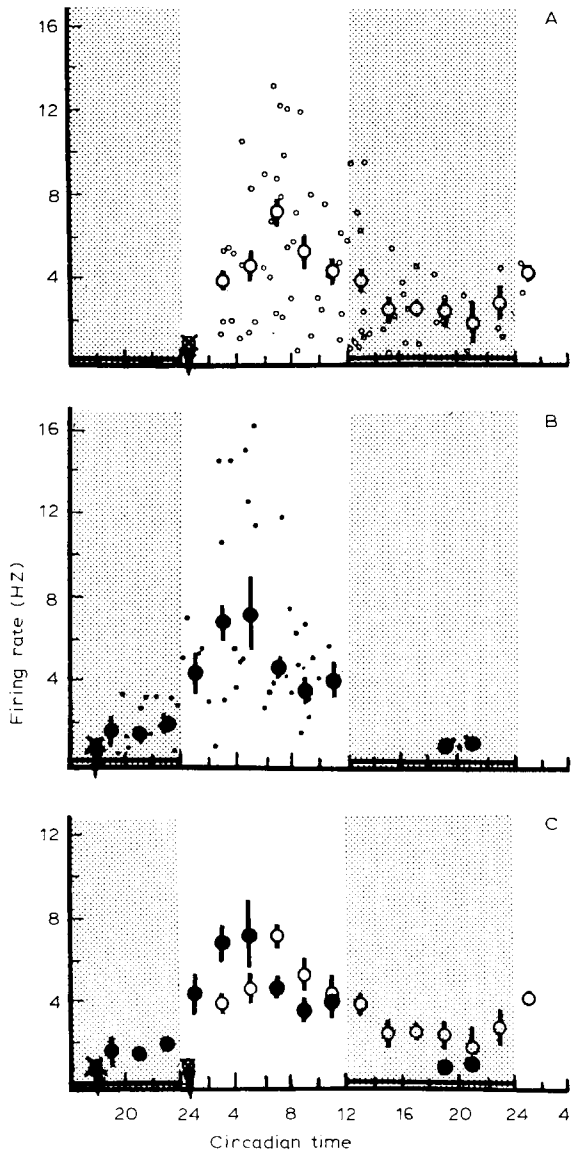

Fig. 1. Single unit firing rates of SCN neurons in hypothalamic brain slices prepared during the light vs dark cycle. Firing rate (Hz) is plotted against the circadian time of the donors, which had been entrained to the same 12L:12D lighting cycle. A: firing rates of all neurons counted to criterion (○, 78 units) as well as mean firing rates (○ ± S.E.M.) for 2 h circadian intervals (mean for CT 2–4 plotted at CT 3) in the SCN of a brain slice prepared at CT 0.5 (✖), one half hour into the donor's day. B: individual firing rates(•, 58 units) and 2 h means (● ± S.E.M.) from SCN isolated in a brain slice at CT 17.8 (✖), nearly 6 h into the donors' night. C: mean firing rates for A and B, plotted together to facilitate direct comparison. Sacrifice and brain slice preparation at CT 17.8, the donors' dark period, advanced the phase of the next oscillation in SCN single unit firing (●, peak = CT 4.5) relative to that for a slice prepared at CT 0.5, during the donor's light period (○, peak = 7.25).

peak of the next electrical oscillation.

For SCN neurons in hypothalamic slices prepared during the donor's day, the oscillation in single unit activity continued unperturbed in phase, with a maximum and period similar to those observed both in animals implanted with chronic multiunit electrodes[12,13] and in previous studies on SCN in vitro[4,5,27]. Fig. 1A presents the results of a typical experiment initiated during the donor's day. The firing rates of 78 single units in a hypothalamic slice isolated at circadian time 0.5 (CT 0.5 = one half hour after 'lights on' in the donor's colony) appear as small open circles. Each point represents the mean of 12–36 10-s counts on a single unit. The averaged means (± S.E.M.) for 2-h intervals are also plotted (large open circles). Peak firing rate for this slice occurred at CT 7.25. Examination of the oscillation in neuronal firing rate from SCN of slices isolated at different points in the light part of the circadian cycle revealed no significant change in the phase of the next oscillation throughout the light period (stars, Fig. 2); the mean peak in single unit firing rate for day-prepared slices was CT 7.87 ± 0.36 h (S.E.M., n = 6 donors).

In contrast, isolation of the SCN during the dark portion of the circadian cycle produced a marked change in the phase of the next oscillation. Firing rates for 58 neurons in an SCN brain slice prepared late in the donor's dark period at CT 17.8 (5.8 h after 'lights out', small closed circles in Fig. 1B), demonstrate that activity rose and peaked earlier than for units from the slice prepared during the day in Fig. 1A. Analysis of 2-h means (large closed circles, Fig. 1B) shows that circadian activity peaked at CT 4.5 in this slice.

Direct comparison of the 2-h means for these two experiments (Fig. 1C) reveals that the phase of the oscillation in firing rate of the slice prepared during the donor's night (large closed circles) was advanced by nearly 3 h relative to that of the slice prepared during the day (large open circles). The earliest time at which the two oscillations clearly diverge is the first point in the subjective day, 7–9 h after the phase-shifting stimulus had been experienced during the night. With the exception of one point at CT 10, the 2-h means near the electrical activity peaks of these two slices do not overlap. Thus, the whole oscillation, not just the peak, has been shifted.

Examination of a range of circadian time points revealed that the phase-shifts in neuronal firing rate expressed in vitro show differential resetting, as do phase-shifts in behavior in vivo. This can be seen by plotting circadian time of SCN isolation in the brain slice against the phase of the subsequent electrical oscillation (stars, Fig. 2). While the donor's light period was phase-neutral, there is a period after the onset of the donor's night when stimulation resulted in delay of the peak of the next oscillation in vitro. Delays of 1.8 and 3.8 h were observed in slices prepared during

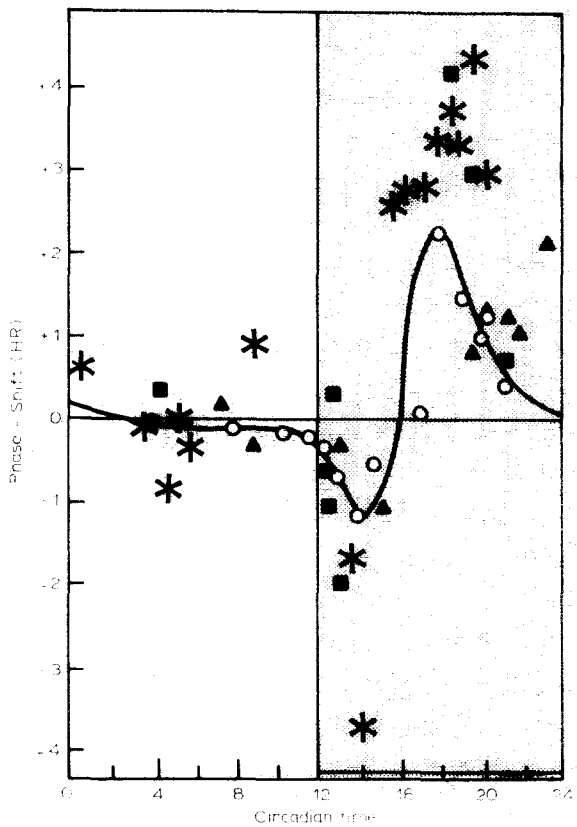

Fig. 2. Phase-response relationship relating phase shifts in neuronal electrical circadian rhythms (*) to the circadian time at which the donor was sacrificed and the brain slice prepared. Each point represents the mean of 50–96 single units sampled over a 12–28 h period in order to define the peak of the oscillations as presented in Fig. 1. For slices prepared during the donors' day, the mean peak is at CT 7.87 ± 0.80 S.D., similar to in vivo[12,13]. For slices prepared during the donors' night, the phase is differentially shifted. These data for phase-shifts in the next peak in firing rate in vitro are similar in pattern to the phase response curve replotted by Rusak and Groos for first cycle shifts in overt behavioral rhythms in DeCoursey's original study (○) of 10-min light-pulses to hamsters maintained in constant darkness[1,24] as well as their own equilibrated behavioral data for hamsters (■) and rats (▲) phase-shifted by direct electrical stimulation of the SCN region[24].

the first 3 h of two donors' dark periods; maximum sensitivity to delay was near CT 14. This was followed by a dramatic change of sign near CT 15 such that subsequent sacrifice and brain slice preparation advanced the peak of the next electrical oscillation up to 4.5 h[3]. Maximum sensitivity to phase advance occurred near CT 19.5. Fig. 2 demonstrates that the pattern of phase-resetting in the isolated SCN in vitro fits very well the classic phase-response curve for intact rodents whose behavioral rhythms are phase-shifted by a 10-min pulse of light[1] (open circles, Fig. 2), as well as the superimposed data for resetting of rodents' behavioral rhythms by direct electrical stimulation of their SCN with implanted electrodes[24] (triangles and squares, Fig. 2).

A striking finding of the present study is that resetting of the neuronal activity rhythm of the isolated SCN shows very large magnitude shifts within only a single cycle. This contrasts with the modest phase-shifts in behavior elicited in intact rodents in one cycle[1] (Fig. 2). It is more similar to the fully-developed phase-shifts in behavioral rhythms of rodents measured several days after stimulation[9,24]. Consideration of the parametric differences between this and other studies indicates that the major difference arises from the isolation of the oscillator in the brain slice. This in turn suggests that the large magnitude first cycle shifts in vitro are a consequence of the removal of negative feedback from other brain regions.

The phase-shifting stimuli in each of the 3 studies represented in Fig. 2 were significantly different. In the behavioral studies, the rhythms of the intact organisms were reset by 10-min light pulses to the eyes or direct electrical stimulation of the SCN during the established dark regime[1,24]. Photic stimulation of the retina activates SCN neurons via the retinohypothalamic pathway of the optic tract[8,15,26]; presumably, direct electrical stimulation of SCN neurons produces the same central effect as this depolarizing input from the eye. In the present study, there are two variables which may have contributed to the observed phase-shift: (1) the brief pulse of light to which the animals were exposed immediately prior to decapitation and (2) the chemical shifts occurring in the brain as a result of decapitation and tissue slicing.

With regard to the first of these, it is unclear that the animals received sufficient light stimulation during slice preparation to alone cause large magnitude shifts. The phase-shifting efficacy of very short (ca. 20–30 s) pulses of light has not been documented in rats, although the duration of longer pulses (ranging from 1 min to 4 h) has been directly correlated with the magnitude of the resulting shift and the slope of the delay/advance transition in the phase-response curve[2,9,11]. The large magnitude of the shifts and steepness of this transition in the present study (Fig. 2) might suggest that a very short light-pulse would not be a sufficiently strong stimulus to fully account for the observed effect, although it could contribute.

Decapitation and the tissue isolation procedure may each cause changes in brain chemistry capable of initiating phase-shifts. Neurons in slices from various brain regions have been reported to experience long-lasting depolarization for about one hour after slice preparation[18]. Such depolarization would be predicted to produce the same central effect of photic stimulation of the eye, as does direct electrical stimulation of the SCN[24]. Massive elevation of extracellular K^+, due to rapid onset of cerebral ischemia upon decapitation[7] and to K^+-release from neurons cut in the slicing procedure[18], may underlie this period of 'spreading depression' of re-excitability. Further, cutting the optic tracts will itself cause a barrage of excitation in the afferents to the SCN. Even the temperature fluctuation of the tissue during the slicing procedure cannot be excluded as a signal for change. Taken together, these considerations suggest that SCN in brain slices prepared at night are exposed to potent phase-shifting stimuli.

Other factors which must be considered in comparing the phase-response relationships in Fig. 2, include species differences as well as the cycle at which the phase-shift is assessed. DeCoursey's original study measured shifts in peak running activity during the first cycle following a 10-min light pulse to hamsters[1]. Although she used a different species of rodent than the present study, and a relatively long pulse of light compared with that experienced by our rats at decapitation, the shifts she measured during the first cycle of wheel-running are modest compared to those we have measured during the first cycle of electrical activity of SCN in vitro. Phase advances in overt behavioral rhythms (e.g. feeding, wheel-running) are reported to gradually shift to a stable, equilibrated phase over several circadian cycles[19,20]. In spite of species and stimulus differences, the shifts we

have measured in the first cycle in vitro are more like the equilibrated shifts reported for hamsters exposed to a 10-min pulse of light[21] than the first cycle changes in vivo.

The second set of data in Fig. 2 are shifts in the phase of both feeding and wheel-running in rats and hamsters respectively, obtained by Rusak and Groos[24] by applying various stimulation regimes to the SCN with implanted electrodes. Behavioral rhythms were allowed to equilibrate for several days before assessing the resulting phase-shift. These procedural differences complicate direct comparison of even this hamster data to DeCoursey's study of first cycle phase shifts stimulated by 10 min of light. Nevertheless, their data fit the shape of the phase-response curve derived from DeCoursey's data with a delay period in the early night followed by a period sensitive to phase advance. The maximum phase delay and advance are greater in the Rusak and Groos study, possibly both because of greater stimulus efficacy and because the full extent of the shifts has been achieved through equilibration. Nevertheless, first cycle shifts in single unit firing rate in the SCN in vitro are already at least as large as these equilibrated behavioral phase shifts which accumulate over days after stimulation of the SCN in intact animals[24].

Thus, the most important difference amongst the studies in Fig. 2 may in fact be that we are studying the resetting of the SCN in isolation and in doing so may have revealed a basic property of the primary oscillator: that it fully resets its phase during the first cycle. This conjecture must be verified by observing several cycles in vitro. Should they be free of transients, maintaining the first cycle shift, it would mean that in vivo, the intrinsic shift of the primary oscillator is damped over several cycles by ongoing interactions with other brain regions which exert negative feedback during each cycle until a new equilibrium is achieved. Regions which oscillate 180° out of phase due to inhibitory inputs from the SCN[12] would be effective sources of feedback loops which would slow the shift toward phase advance. The SCN in the hypothalamic slice has been removed from these inertial forces and thus is able to shift more fully in one cycle. Therefore, regardless of which stimuli initiate the shift, these data support the prediction of Pittendrigh that the clock is immediately reset[19,20].

The present study clearly demonstrates that the effect of a resetting stimulus which occurs at the time of slice preparation is preserved in the SCN in vitro. The faithfulness of the neuronal phase-shift data from the isolated SCN to the sign and shape of the behavioral phase-response curve for intact animals allows us to assume that the mechanism of readjustment of the primary oscillator is fundamentally the same and establishes the usefulness of the SCN in vitro for study of this mechanism.

This finding may cause some reinterpretation of in vitro studies of SCN neurophysiology in which brain slices were prepared during the donor's dark period. The neuronal activity studied under these conditions most probably represents the phase-shifted condition.

By establishing the sensitivity of the SCN to phase-shifting during the dark phase, phase-neutral points in the cycle can be selected for initiating experiments designed to probe the mechanism of the oscillator in the unperturbed circadian system in isolation. Finally, these data provide a timeframe for examining direct effects on the oscillator of putative phase-shifting agents, including those not testable in vivo due to their toxicity to the organism or the difficulty of specific application to the SCN in situ.

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1 DeCoursey, P.J., Function of a light response rhythm in hamsters, *J. Comp. Cell. Physiol.*, 63 (1964) 189–196.
2 Gander, P.H. and Lewis, R.D., Phase-resetting action of light on the circadian activity rhythm of *Rattus exulans*, *Am. J. Physiol.*, 245 (1980) R10–R17.
3 Gillette, M.U., Preparation of brain slices from the suprachiasmatic nuclei of rat can reset the circadian clock, *J. Physiol. (London)*, 369 (1985) 55P.
4 Green, D.J. and Gillette, R., Circadian rhythm of firing rate recorded from single cells in the rat suprachiasmatic brain slice, *Brain Research*, 245 (1982) 198–200.
5 Groos, G. and Hendriks, J., Circadian rhythms in electrical

discharge of rat suprachiasmatic neurones recorded in vitro, *Neurosci. Lett.*, 34 (1982) 283–288.
6 Hatton, G.I., Doran, A.D., Salm, A.K. and Tweedle, C.D., Brain slice preparation: hypothalamus, *Brain Res. Bull.*, 5 (1980) 405–414.
7 Hatton, G.I., Hypothalamic neurobiology. In R. Dingledine (Ed.), *Brain Slices*, Plenum Press, New York, 1984, pp. 345.
8 Hendrickson, A.E., Wagoner, N. and Cowan, W.M., An autoradiographic and electron microscope study of retinohypothalamic connections, *Z. Zellforsch. Mikrosk.*, 135 (1972) 1–26.
9 Honma, K., Honma, S. and Hiroshige, T., Response curve, free-running period and activity time in circadian locomotory rhythm of rats, *Jap. J. Physiol.*, 35 (1985) 643–658.
10 Ibuka, N., Inouye, S.T. and Kawamura, H., Analysis of sleep–wakefulness rhythms in male rats after suprachiasmatic nucleus lesions and ocular enucleation, *Brain Research*, 122 (1977) 33–47.
11 Illnerová, H. and Vaněček, J., Two-oscillator structure of the pacemaker controlling the circadian rhythm of N-acetyltransferase in the rat pineal gland, *J. Comp. Physiol.*, 145 (1982) 539–548.
12 Inouye, S.-I.T. and Kawamura, H., Persistence of circadian rhythmicity in a mammalian hypothalamic 'island' containing the suprachiasmatic nucleus, *Proc. Natl. Acad. Sci. U.S.A.*, 76 (1979) 5962–5966.
13 Inouye, S.-I.T. and Kawamura, H., Characteristics of a circadian pacemaker in the suprachiasmatic nucleus, *J. Comp. Physiol.* 146 (1982) 153–160.
14 Moore, R.Y. and Eicher, V.B., Loss of a circadian adrenal corticosterone rhythm following suprachiasmatic lesion in the rat, *Brain Research*, 42 (1972) 201–206.
15 Moore, R.Y. and Lenn, N.J., A retinohypothalamic projection in the rat, *J. Comp. Neurol.*, 146 (1972) 1–14.
16 Nagai, K., Nishino, T., Nakagawa, N., Nakamura, S. and Fukuda, T., Effect of bilateral lesions of the suprachiasmatic nuclei on circadian rhythm of food intake, *Brain Research*, 142 (1978) 384–389.
17 Nakagawa, H., Hagai, K., Kida, K. and Nishino, T., Control mechanisms of circadian rhythms of feeding behavior influenced by food intake. In M. Suda, O. Hayaishi and H. Nakagawa (Eds.), *Biological Rhythms and their Central Mechanisms*, Naito Foundation Symposium, Elsevier, Amsterdam, 1979, pp. 283–294.
18 Nicholson, C. and Kraig, R.P., The behavior of extracellular ions during spreading depression. In T. Zeuthen (Ed.), *The Application of Ion-Selective Electrodes*, Elsevier, Amsterdam, 1981, pp. 226–228.
19 Pittendrigh, C.S., Circadian oscillations in cells and the circadian organization of multicellular systems, In F.O. Schmitt and F.G. Worden (Eds.), *The Neurosciences, Third Study Program*, MIT Press, Cambridge, MA, 1974, p. 444.
20 Pittendrigh, C.S., Circadian organization and photoperiodic phenomena, In B.K. Follett (Ed.), *Biological Clocks in Reproductive Cycles*, J. Wright, Bristol, 1983.
21 Pittendrigh, C.S. and Daan, S., A functional analysis of circadian pacemakers in nocturnal rodents. IV. Entrainment: pacemaker as clock, *J. Comp. Physiol.*, 106 (1976) 291–331.
22 Raisman, G. and Brown-Grant, K., The 'suprachiasmatic syndrome': endocrine and behavioral abnormalities following lesions of the suprachiasmatic nuclei in the female rat, *Proc. Royal Soc. London, Ser. B*, 198 (1977) 297–314.
23 Richter, C.P., Sleep and activity: their relation to the 24 h clock, *Proc. Assoc. Res. Ment. Disease*, 45 (1967) 8–27.
24 Rusak, B. and Groos, G., Suprachiasmatic stimulation phase-shifts rodent circadian rhythms, *Science*, 215 (1982) 1407–1409.
25 Saleh, M.A. and Winget, C.M., Effect of suprachiasmatic lesions on diurnal heart rate rhythm in the rat, *Physiol. Behav.*, 19 (1977) 561–564.
26 Sawaki, Y., Retinohypothalamic projection: electrophysiological evidence for the existence in female rats, *Brain Research*, 120 (1977) 336–341.
27 Shibata, S., Oomura, Y., Kita, H. and Hattori, K., Circadian rhythmic changes of neuronal activity in the suprachiasmatic nucleus of the rat hypothalamic slice, *Brain Research*, 247 (1982) 154–157.
28 Stephan, F.K. and Kovacevic, N.S., Multiple retention deficit in passive avoidance in rats is eliminated by suprachiasmatic lesions, *Behav. Biol.*, 22 (1978) 456–460.
29 Stephan, F.K. and Nunez, A.A., Elimination of circadian rhythms in drinking, activity, sleep and temperature by isolation of the suprachiasmatic nuclei, *Behav. Biol.*, 20 (1977) 1–16.
30 Stephan, F.K. and Zucker, I., Circadian rhythms in drinking behavior and locomotor activity of rats are eliminated by hypothalamic lesions, *Proc. Natl. Acad. Sci. U.S.A.*, 69 (1972) 1583–1586.
31 Wansley, R.A. and Halloway, F.A., Lesions of the suprachiasmatic nucleus (hypothalamus) alter the normal oscillation of retention performance in the rat, *Soc. Neurosci. Abstr.*, 1 (1975) 522.