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Circadian rhythm of the rat suprachiasmatic brain slice is rapidly reset by daytime application of cAMP analogs

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Cellular mechanisms underlying the primary circadian pacemaker in mammals were investigated by isolating rat suprachiasmatic nuclei in brain slices and maintaining them in vitro for up to 3 days. The circadian rhythm of neuronal firing rate was used to assess the phase of the pacemaker. This rhythm was rapidly reset by bath application of cAMP analogs. Moreover, the pacemaker demonstrated circadian sensitivity to analog treatment: the rhythm was advanced by application during the donor's day, but not during the donor's night. These results suggest that cAMP-mediated events may stimulate pacemaker afferents within the SCN or may directly influence the pacemaker mechanism.

Embedded in the mammalian brain is a biological clock that generates daily oscillations in its own activity, even in the absence of external timing cues. The oscillations of this endogenous pacemaker time the circadian rhythms of the organism's behavior and physiology[27]. The pacemaker is located within the suprachiasmatic nuclei (SCN) of the hypothalamus[20,28]. While the SCN are very small (0.068 mm^3)[30], paired nuclei at the base of the brain, they have numerous interconnections with other brain regions[15,20,31,32]. The size, location and connections of the SCN make it difficult to study their endogenous cellular properties in vivo. However, the pacemaker survives intact in the hypothalamic brain slice[13] where it is accessible to experimental approaches aimed at dissecting cellular mechanisms.

As an early step in elucidating these mechanisms, we tested the ability of cAMP analogs to alter the phase of an endogenous circadian oscillation of SCN maintained in a brain slice. A regulatory pathway integral to a variety of neuronal oscillators involves cAMP[11,16], and cAMP has been implicated in at least one other circadian clock[6]. We found that the endogenous circadian rhythm in SCN neuronal firing rate was rapidly shifted to a new stable phase by analogs stimulating cAMP-dependent pathways. This may be the result of altering the temporal order in specific pathways that are either part of the oscillator's structure or have access to it. Although there have been preliminary indications[4,9,24], this study definitively establishes that the mammalian circadian pacemaker can be reset in vitro.

Hypothalamic brain slices were prepared from 2–5-month-old Long–Evans rats which had been bred and reared in our colony. They were housed from birth on a 12 h light:12 h dark schedule. Donors were sacrificed and brain slices prepared only during the light portion of their circadian cycle, as preparation during the animal's night induces phase-shifts in the rhythms of SCN neurons[7,8]. Coronal slices (500 μm thick) of the hypothalamic region containing the SCN were cut with a manual tissue chopper. Slices were placed in a brain slice chamber[14] with a 14 ml bath volume at the interface of a humid 95% O_2:5%

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CO_2 atmosphere and Earle's Balanced Salt Solution (GIBCO) supplemented with 24.6 mM glucose and 26.2 mM bicarbonate[8,25]. They were maintained at 37 °C in continuous light and perifused at the rate of 34 ml/h.

Our methods of random sampling, extracellular recording and counting of single neuronal units have been reported previously[8]. The pattern of electrical activity of the population of single neuronal units was recorded in a single slice and analyzed using the running average derived from 1 h overlaps of 2 h means. The time of the peak in the electrical activity rhythm was determined by visual inspection of the oscillation for the symmetrically highest point[25].

SCN in vitro continue the circadian pattern of neuronal activity recorded in vivo[15] unperturbed by the trauma of surgery[7,8,13]. Activity recorded continuously during the first 24 h in culture (Fig. 1A, replotted[8]) rises above the daily mean during the donor's day, peaks at circadian time 7.25 (CT 7.25, where CT starts when lights go on in the donor colony and continues for 24 h before starting over at CT 0 at the next lights on), and falls below the daily mean firing rate during the donor's night. We have shown that correspondence of this pattern of oscillation to the donor's light cycle is the same in slices prepared early (CT 0.5) through late (CT 10) in the donor's day, as well as for slices from animals maintained on a reversed light cycle[10].

For SCN maintained in vitro for longer periods (n = 3), activity on days 2 and 3 consistently peaked near CT 7.0 with a standard error of less than 0.5 h (Fig. 1B; ref. 25), in close agreement with the time-of-peak established for the first cycle. The observations that the SCN pacemaker generates repeated circadian oscillations when isolated from the organism and that its period is stable in our conditions of culture allowed us to use the time-of-peak to measure the phase of the pacemaker.

In order to test the hypothesis that cAMP pathways might access or comprise a part of the pacemaker mechanism, cAMP analogs were applied directly to the slices. At various times after tissue preparation, perifusion was stopped for 1 h and the medium was replaced either with fresh medium or medium that had been made 5×10^{-4} M with 8-benzylamino-cAMP (BA-cAMP, Sigma) or 8-parachlorophenylthio-cAMP (CPT-cAMP, ICN). These membrane-soluble analogs of cAMP activate cAMP-dependent protein kinases yet resist phosphodiesterase degradation[19] and thus are extremely effective at stimulating cAMP-dependent pathways. cAMP analog was dissolved in fresh medium, the pH was adjusted to 7.2, and the solution was bubbled with 95% O_2:5%

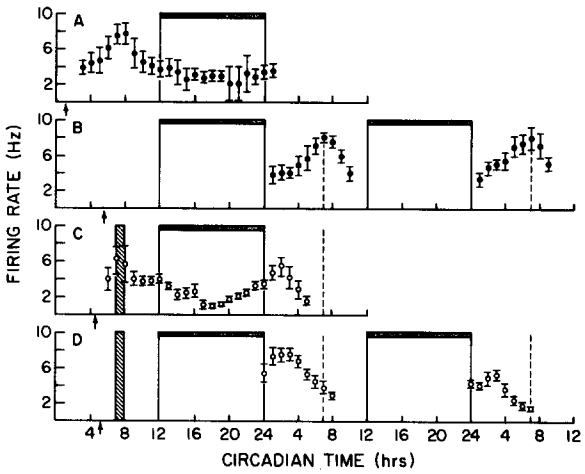

Fig. 1. Circadian patterns of the population of SCN neuronal activities in unperturbed and BA-cAMP-treated brain slices. Each panel represents a different experiment; data for each panel are from a single SCN. A: the circadian pattern of neuronal activity recorded for a complete 24 h period from an unperturbed SCN (data replotted from ref. 8). Activity is high in the donor's day, peaking at CT 7.25, and low during the donor's night (the interval marked with a horizontal bar). B: circadian activity measured in a single SCN during the donor's day on days 2 and 3 in vitro. Neuronal activities peaked at CT 7.0 (marked with vertical dashed lines); these peaks are in close temporal agreement with the time of peak activity in SCN studied during their first cycle in vitro (A). C: treatment of the SCN with cAMP analog during CT 7.0–8.0 changes the phase of the next oscillation. The SCN were pulsed with medium supplemented with 5×10^{-4} M BA-cAMP during the 1 h period designated by the vertical cross-hatched column. Activity continued to oscillate through the first daily high and nightly low period, then rose and peaked early on the second day in culture. Peak activity occurred at CT 2.2. The relationship of the time of this peak to the control peak can be assessed by comparing it to the dashed vertical line which indicates the time of the mean peak in untreated slices (B). This is a 4.8 h advance. D: in order to determine whether this change in phase was a transient perturbation or a true shift in the clock's pacemaker, activity was followed in a single SCN for two cycles after BA-cAMP treatment. In this experiment, activity on the second day peaked at CT 2.2 and at CT 2.5 on day 3. The close temporal agreement of these peaks indicates that the phase change induced in day 2 is apparently complete and is the result of resetting of the pacemaker mechanism. In each of the above experiments, brain slices were prepared at the point designated in each experiment by a vertical arrow. The average firing rate ± S.E.M. of all units sampled is plotted in overlapping 2 h bins (composed of 4–10 individual mean neuronal firing rates from a single SCN) to produce a running average.

CO_2 and warmed to 37 °C for >5 min before complete exchange of the volume in the brain slice chamber was made. This assured that the tissue experienced very little change except for exposure to the cAMP analog. After 1 h the medium was replaced with normal medium from the reservoir and perifusion was resumed (control pulse and BA-cAMP) or the medium was exchanged by continued perifusion (CPT-cAMP).

In experiments where brain slices were pulsed on day 1 with either normal medium or BA- or CPT-cAMP, the phase of the pacemaker was assessed by determining the time of peak activity during the day(s) after treatment. This avoided the possible confounding of transient changes due to direct effects of cAMP analogs upon neuronal excitability[12,17] with shifts in the phase of the underlying pacemaker. Phase-shifts were determined by comparing the time-of-peak in treated slices with untreated slices maintained in vitro for the same period of time, and significance determined using Student's *t*-test.

Whereas replacement of the perifusion medium with normal medium for 1 h during the donor's day had no effect on the time-of-peak of the next electrical oscillation in vitro (0.63 ± 0.18 h phase-advance, $n = 2$), daytime replacement with medium containing either BA-cAMP or CPT-cAMP induced a significant change in the phase of the next oscillation ($P < 0.01$, $n = 18$). In two representative experiments (Fig. 1C,D), exposure between CT 7.0–8.0 caused a 4.8 h advance of the subsequent peak in firing rate compared with untreated controls. This phase-shift was perpetuated without augmentation during the next cycle (Fig. 1D; ref. 25), so that the peak on day 3 occurred nearly 24 h after that on day 2 ($n = 3$), but again more than 4 h earlier than in controls. These findings show that the isolated pacemaker can be reset very rapidly in vitro: the large shift induced by cAMP analogs appears to be completed during the first cycle after treatment and is stable thereafter. This allowed us to use the time-of-peak in the first cycle after treatment to assess the degree of phase-shift.

Differential day–night sensitivity to phase-shifting stimuli is a characteristic of circadian systems[2,3,5,23,27]. We therefore compared the sensitivity of the SCN in the brain slice to cAMP analogs applied in the donor's day vs night. One-hour treatments with BA-cAMP during the donor's midday (CT 3.0–9.0) advanced the time of peak activity by 4.27 ± 0.27 h ($n = 16$) (Fig. 2). Significant phase-advances were also induced by CPT-cAMP treatment during the donor's day. Although the protocol differed in that the 1 h pulse was followed by a gradual wash-out phase, exposure at CT 7.5 or 6.75 resulted in phase-advances of 2.8 and 2.5 h, respectively. Conversely, 1 h treatment with BA-cAMP between CT 17.0.0–23.0, during the donor's night, was ineffective at shifting the peak (+0.13 ± 0.14 h phase-advance, $n = 4$) (Fig. 2).

The results of this study provide new documentation of the intrinsic pacemaking properties of the SCN. That is, SCN maintained in vitro in a hypothalamic slice continue to generate a circadian rhythm of neuronal firing rate for at least three days. The rhythm is stable and truly endogenous: the SCN apparently require little more than an energy supply and a moist, buffered, high oxygen environment to generate each 24 h oscillation. Further, the resetting mechanism must be intrinsic to the SCN: the endogenous circadian rhythm can be rapidly reset to a stable new phase by chemical treatment in vitro.

In addition, the results point to a potential role in SCN phase-shifting for cAMP. The ability of two structurally different cAMP analogs to produce similar effects suggests that these agents work through stimulating cAMP-dependent pathways. Similar phase-shifting by different classes of chemical agents that enhance endogenous cAMP levels further supports the notion that cAMP-stimulated pathways are

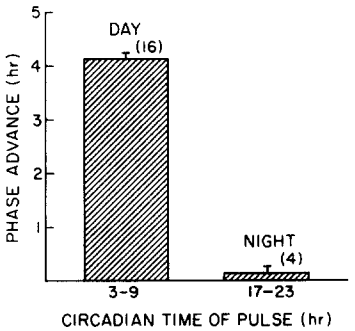

Fig. 2. Relationship between the time of the pulse with BA-cAMP and the time of peak activity in the subsequent oscillation in firing rate of SCN neurons. One hour pulses during the donor's midday (CT 3.0–9.0, $n = 16$) produced a mean phase advance of 4.27 h, while 1 h pulses between CT 17.0–23.0 ($n = 4$), the donor's night, had no effect on the timing of the next oscillation.

part of an SCN phase-shifting mechanism[25].

The phase-dependent effect of 1 h pulses of BA-cAMP upon the circadian oscillation in SCN electrical activity in vitro differs from the classical phase-responsiveness of rodent behavioral cycles to 15 min pulses of light[3] or to direct electrical stimulation of the SCN in vivo[26]. The phase-advances induced by cAMP analogs appear to be 180° out of phase with those induced by light pulses or electrical stimulation.

Rather, the circadian timing of phase changes induced by cAMP analogs more closely resembles the sensitive period for a group of agents that are only effective during the circadian day in vivo: dark pulses on a background of environmental light[2,5], injection of neuropeptide Y[1] or glutamate[18] into the SCN as well as peripheral injection of the protein synthesis inhibitor, anisomysin[29]. Each treatment effective during the day could potentially act through a common pathway with cAMP.

Because cAMP analogs are able to induce phase-shifting of the SCN in a brain slice in vitro, cAMP must act within the SCN. There cAMP-mediated events may be involved in either an input to or as part of the pacemaker. To determine that a phase-shifting agent is a component of a biological oscillator, it must also normally oscillate at a fixed phase angle and be shifted to the same phase angle by resetting events. Documentation of appropriate and cyclic fluctuation of cAMP in the SCN of intact rats[21] coupled with the potent phase-shifting effects we report here strengthens the likelihood that cAMP is part of an integral regulatory pathway of the mammalian circadian pacemaker. The detailed relationship between cAMP and the SCN pacemaker remains to be determined.

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