Journal of Physiology (1992), **450**, *pp.* 629–642
With 5 figures
Printed in Great Britain

SEROTONIN REGULATES THE PHASE OF THE RAT SUPRACHIASMATIC CIRCADIAN PACEMAKER *IN VITRO* ONLY DURING THE SUBJECTIVE DAY

BY MARIJA MEDANIC AND MARTHA U. GILLETTE*†

*From the Department of Physiology and Biophysics, University of Illinois, 407 S. Goodwin Ave., Urbana, IL 61801, USA and the * Departments of Cell and Structural Biology, Physiology and Biophysics and The Neuroscience Program, University of Illinois, 505 South Goodwin Avenue, Urbana, IL 61801, USA*

(*Received 1 August 1991*)

SUMMARY

1. The suprachiasmatic nucleus (SCN) of the hypothalamus is the primary pacemaker for circadian rhythms in mammals. The 24 h pacemaker is endogenous to the SCN and persists for multiple cycles in the suprachiasmatic brain slice.

2. While serotonin is not endogenous to the SCN, a major midbrain hypothalamic afferent pathway is serotonergic. Within this tract the dorsal raphe nucleus sends direct projections to the ventrolateral portions of the SCN. We investigated a possible regulatory role for serotonin in the mammalian circadian system by examining its effect, when applied at projection sites, on the circadian rhythm of neuronal activity in rat SCN *in vitro*.

3. Eight-week-old male rats from our inbred colony, housed on a 12 h light : 12 h dark schedule, were used. Hypothalamic brain slices containing the paired SCN were prepared in the day and maintained in glucose and bicarbonate-supplemented balanced salt solution for up to 53 h.

4. A 10^{-11} ml drop of 10^{-6} M-serotonin (5-hydroxytryptamine (5-HT) creatinine sulphate complex) in medium was applied to the ventrolateral portion of one of the SCN for 5 min on the first day *in vitro*. The effect of the treatment at each of seven time points across the circadian cycle was examined. The rhythm of spontaneous neuronal activity was recorded extracellularly on the second and third days *in vitro*. Phase shifts were determined by comparing the time-of-peak of neuronal activity in serotonin- *vs.* media-treated slices.

5. Application of serotonin during the subjective day induced significant advances in the phase of the electrical activity rhythm ($n = 11$). The most sensitive time of treatment was CT 7 (circadian time 7 is 7 h after 'lights on' in the animal colony), when a $7{\cdot}0 \pm 0{\cdot}1$ h phase advance was observed ($n = 3$). This phase advance was perpetuated on day 3 *in vitro* without decrement. Serotonin treatment during the subjective night had no effect on the timing of the electrical activity rhythm ($n = 9$).

6. The specificity of the serotonin-induced phase change was assessed by treating

† To whom correspondence should be addressed.

slices in the same manner with a microdrop of serotonergic agonists, 5-carboxamidotryptamine, that targets the 5-HT_1 class of receptors, or 8-hydroxy-dipropylaminotetralin (8-OH DPAT), that acts on the 5-HT_{1A} receptor subtype. Daytime (CT 9) application of 5-carboxamidotryptamine resulted in a $6{\cdot}0 \pm 0{\cdot}1$ h phase advance ($n = 3$), while treatment during the subjective night (CT 15, $n = 2$) had little observable effect. Similarly, treatment with 8-OH DPAT at CT 9 induced a phase advance of $6{\cdot}9 \pm 0{\cdot}1$ h ($n = 3$) in the rhythm of electrical activity.

7. These results demonstrate that serotonin can induce large phase changes in the SCN circadian pacemaker and that the SCN undergoes endogenous changes in sensitivity to serotonin. The data suggest that serotonergic inputs to the ventrolateral SCN can regulate the circadian pacemaker during the daytime.

INTRODUCTION

Substantial evidence points to the suprachiasmatic nuclei (SCN) as the endogenous pacemaker that regulates the timing of most circadian rhythms of behaviour, physiology and metabolism in mammals. The SCN generate circadian patterns of neural activity (Inouye & Kawamura, 1979) and glucose utilization (Schwartz & Gainer, 1977; Schwartz, Davidsen & Smith, 1980) in various brain regions *in vivo*. Near-24 h rhythms continue in surgically isolated SCN but are abolished in other brain regions by deafferentation from SCN (Inouye & Kawamura, 1979). The nuclei are capable of sustaining rhythmicity of endogenous neuronal firing (Gillette & Prosser, 1988) and peptide secretion (Earnest & Sladek, 1987) for multiple cycles *in vitro*, under constant conditions devoid of any external time cues.

Green & Gillette (1982) showed that the endogenous circadian rhythm of electrical activity (measured by extracellular recording) present in SCN of intact organisms is preserved in SCN within a hypothalamic brain slice. The waveform and the time-of-peak in this oscillation were found to be unaffected by day-time preparation of brain slices (Gillette, 1986; Gillette & Reppert, 1987). The robustness, uniformity and stability of the waveform over multiple cycles *in vitro* permit use of this oscillation to monitor the underlying activity of the pacemaker in isolation from inputs and modifiers (Prosser & Gillette, 1989). We used this *in vitro* preparation to examine regulation of circadian timing by exogenous serotonin.

Photic changes in the external world are primary regulators of circadian timing. The retino-hypothalamic tract and the geniculo-hypothalamic tract form the visual pathways of entrainment. The retino-hypothalamic tract carries light information directly from the retina to the ventrolateral portion of the SCN (Moore & Lenn, 1972). Although the transmitter in the retino-hypothalamic tract has not been completely identified, it is thought to influence the SCN via the excitatory amino acid glutamate (Liou, Shibata, Iwasaki & Ueki, 1986; Cahill & Menaker, 1987). The geniculo-hypothalamic projection forms a secondary visual input from the lateral geniculate nucleus to the SCN (Swanson, Cowan & Jones, 1974; Card & Moore, 1982). This tract also relays information about the lighting regime, but is thought to be involved in mediating the effects of dark pulses (Harrington & Rusak, 1988). Terminals of this neuropeptide Y-containing tract are overlapping those of the retinal pathways (Card & Moore, 1982; Groos, Mason & Meijer, 1983).

Projections from the midbrain raphe nuclei form a distinctive pathway leading to

the SCN. While the functional nature of these projections has not been elucidated, it can be hypothesized that they play a modulatory role. The raphe projects to the ventrolateral portions of the rostral SCN (Aghajanian, Bloom & Sheard, 1969; van den Pol & Tsujimoto, 1985), as well as to the ventral lateral geniculate nucleus (Moore, Halaris & Jones, 1978). Its serotonergic terminals in the SCN overlap with those directly regulated by photic stimulation, the retino-hypothalamic and geniculo-hypothalamic inputs (Ueda, Kawata & Sano, 1983; Guy, Bosler, Dusticier, Pelletier & Calas, 1987). The pathway from the raphe to the SCN is thought to be activated by arousal, possibly through motor activity stimulated by the onset of darkness (Mrosovsky & Salmon, 1987).

Serotonin is a widely distributed neurotransmitter that in many cases causes suppression of spontaneous firing rates of neurons. This is true in the case of the SCN. Stimulation of the raphe *in vivo* results in an overall inhibition of firing rates of SCN neurons (Groos *et al.* 1983). Similarly, microionophoretic application of serotonin to the SCN has an inhibitory effect on the firing rate of the majority of neurones, both *in vitro* and *in vivo* (Mason, 1986; Meijer & Groos, 1988).

Interestingly, SCN neurons in brain slices show a circadian variation in sensitivity to serotonin in terms of firing rate, such that the neurons are more sensitive to inhibition of firing rate by serotonin in the subjective night (Mason, 1986). Besides the circadian rhythm of sensitivity to serotonin, the SCN also display a circadian rhythm in recovery from serotonin. Mason (1986) demonstrated that the time of recovery after inhibition of neuronal activity was longer when serotonin was administered during the day-time than during the night.

Serotonin receptors are abundantly distributed throughout the SCN (van den Pol & Tsujimoto, 1985). These receptors are capable of serotonin reuptake in a temporally dependent manner, with higher rates of reuptake during the subjective night (Meyer & Quay, 1976). Intragastric administration of imipramine, a serotonin reuptake blocker, was found to lengthen and enhance the inhibitory action of serotonin in the SCN (Meijer & Groos, 1988). Studies by Wirz-Justice, Krauchi, Morimesa, Willener & Feer (1983) demonstrated that serotonin receptors in the SCN have a temporal sensitivity to imipramine binding which peaks during the night phase in the rat.

Serotonergic inputs are not required for sustaining endogenous circadian organization of the pacemaker. Application of 5,7-dihydroxytryptamine, which selectively lesions serotonergic inputs, does not affect the period of oscillator *in vivo* (Honma, Watanabe & Hiroshige, 1979; Smale, Michels, Moore & Morin, 1990). Also complete lesions of the raphe have little effect on the entrainability of the animal by light (Kam & Moberg, 1977). However studies on activity-stimulated phase shifts of locomotor activity in the day (Mrosovsky & Salmon, 1987) and the circadian nature of serotonin sensitivity in the SCN suggest possible involvement of serotonin in day-time regulation of the circadian pacemaker.

In this study we directly tested the hypothesis that serotonin has a regulatory role in the SCN. Serotonin was briefly and focally applied to the region of raphe inputs to the rat SCN in brain slices. Effects of serotonin application at different phases of the circadian cycle were determined by measuring the rhythm of neuronal activity on the second and third day after treatment. The specificity of the serotonin-induced phase shifts was assessed by treatment with agonists 5-carboxamidotryptamine and

8-hydroxy-dipropylaminotetralin (8-OH DPAT), which are specific for 5-HT_1 and 5-HT_{1A} receptor subtypes, respectively. We found that serotonin has a strong phase-shifting effect in the middle of the day, but has no effect when applied during the night.

METHODS

Preparation of brain slices

Male Long–Evans rats from our inbred colony were used in this study. Animal care and brain slice preparation were performed humanely, in accordance with guidelines from the American Veterinary Medical Association. The animals were kept on a schedule of 12 h of light, and 12 h of darkness, with food and water available *ad libitum* from birth to 8 weeks, when they were studied. The animals were killed during the 'lights on' period of the 24 h cycle, 1–10 h before serotonin application. This was necessary to avoid phase-shifting effects which have been shown to occur with manipulations in the night-time (Gillette, 1986). The animals were gently introduced into the guillotine, decapitated, and the brain was quickly dissected from the skull. The brain was then manually sectioned to form a block of tissue containing the hypothalamic region. This block of tissue was transferred to a mechanical tissue chopper where 500 μm in coronal slices were made. The hypothalamic slices containing the SCN (clearly visible under the microscope) were reduced even further under the microscope by cutting away excess hypothalamic tissue, and then transferred to the brain slice dish where they were maintained for up to 3 days. This procedure was performed within 5 min from the time of decapitation to avoid development of irreversible hypoxic conditions. A diagram of the hypothalamic brain slice used in this study can be seen in Fig. 1.

The brain slice dish, consisting of an inner and an outer chamber, was modelled after Hatton, Doran, Salm & Tweedle (1980). The outer chamber, a water-bath that provides a constant environment for the slices, was filled with double-distilled water warmed to 37 °C and bubbled with 95 % O_2, 5 % CO_2 to provide a moist, high-oxygen atmosphere at the surface of the slice. The inner chamber was made up of a central and an outer well that were continuously perfused with supplemented salt solution at a rate of 30 ml/h. The medium consisted of Earle's Balanced Salt Solution (0·2 g/l $CaCl_2$, 0·4 g/l KCl, 0·0977 g/l $MgSO_4$, 6·8 g/l NaCl and 0·14 g/l $NaH_2PO_4.H_2O$; GIBCO), supplemented with 24·6 mM-glucose and 26·2 mM-$NaHCO_3$, warmed in a reservoir to 39 °C at a pH of 7·20 and oxygenated by a gas mixture of 95 % O_2 and 5 % CO_2 (which adjusted the pH at 7·40). The temperature of the medium around the brain slices in the central well was $37 \pm 0{\cdot}1$ °C.

The slices were placed on a fibre mesh (Mr Coffee® filter) that covered the central well of the inner chamber of the dish. The medium level was adjusted to come up and around the tissue to bathe the slices without floating them; the slices rested at the interface of the medium and the atmosphere. The tissue was illuminated by a fibre-optics lamp throughout the experiment.

Experimental treatments

Serotonin (5-hydroxytryptamine creatinine sulphate, Sigma, USA) or serotonergic agonists, 5-carboxamidotryptamine (RBI) and 8-hydroxy-dipropylaminotetralin (8-OH DPAT, RBI), were applied to the ventrolateral portion of one of the paired SCN in the slice (Fig. 1), for 5 min at various times across the circadian cycle. Fifteen minutes before the treatment, a 10^{-6} M-solution of serotonin in supplemented salts medium was prepared, and warmed and oxygenated for ~ 2 min. Silanized micropipettes (tip = 2 μm) were smoothed by fire-polishing the large end, back-filled with the 10^{-6} M-serotonin solution, and fitted into the end of 1 m length of Teflon tubing. The tubing was filled with distilled water and attached to a syringe. The micropipette connected to this delivery apparatus was fitted on a micromanipulator and advanced, under microscopic guidance, until it was above the place of delivery, on the ventrolateral SCN. Perfusion of medium throughout the chamber was stopped. A microdrop was created by pressing down on the syringe plunger to form a drop 3–4 μm in diameter, and then gently pulling back to prevent the drop from enlarging further. To deliver the microdrop the micropipette was advanced until the drop made contact with the surface of the slice. After 5 min, the slice was manually rinsed with medium in a direction away from the rest of the SCN (see Fig. 1).

Test runs prior to the actual experiments were performed with 0·1 % Methylene Blue in medium to determine the extent of drop spreading. These drops were confined to a small area within the ventrolateral region of the SCN. The estimated volume of a typical microdrop was 3×10^{-11} ml.

This was calculated from the volume of a spherical drop with a 3–4 μm diameter. This diameter was directly measured by an ocular micrometer on test drops generated with a similar pressure to that in actual experiments, from silanized micropipettes with 2 μm tips, and is representative of the size of experimental microdrops. When the microdrop touches the surface of the slice, the serotonin becomes diluted immediately. Thus, the effective concentration of serotonin at the receptor sites is considerably less than 10^{-6} M, but cannot be determined directly.

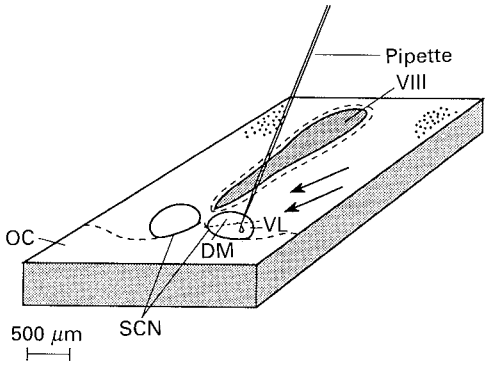

Fig. 1. Diagrammatic illustration of the microdrop application technique on a 500-μm-thick coronal hypothalamic brain slice. The suprachiasmatic nuclei (SCN) are located on either side of the third ventricle (VIII) and dorsal to the optic chiasm (OC). The micropipette is positioned directly above the ventrolateral region of one SCN (VL denotes ventrolateral, and DM dorsomedial). Arrows indicate the direction of media flow rinsing the serotonin treatment off the slice.

Agonists were similarly applied, at 10^{-6} M concentrations, with the only procedural difference being that the 8-OH DPAT solution was made in unoxygenated solution due to the instability of this agonist in oxygen.

To verify that the observed phase shifts were due to serotonin treatment rather than the mechanical or thermal perturbations during treatment, experimental treatments were compared with controls in which a microdrop of medium alone was applied in the same way as in the experiments, to the ventrolateral portions of one of the SCN in a brain slice.

Electrical recording technique

In order to distinguish the effects of the treatment on the pacemaker from acute effects on neuronal activity, extracellular recordings of spontaneous neuronal activity were initiated with the onset of what would be the subjective day in the rat, on the second and third day *in vitro*. This was 11–50 h after application of the microdrop of serotonin to the SCN. We have previously established that the SCN in brain slice generates a complete sinusoidal oscillation in the firing rate of the ensemble of neurons (Gillette, 1986). To facilitate recording for long periods, only that portion of the day surrounding the expected and anticipated peak was studied.

A silver chloride-coated ground wire was placed through the filter mesh, while glass microelectrodes (tip = 2 μm), back-filled with 5 M-NaCl, were used to record electrical activity. The recording electrode was fitted in a micromanipulator and positioned over the slice so that it was in contact with the surface of the tissue. A Narashige MO-8 hydraulic microdrive was used to further advance the electrode through the tissue. The signal picked up by the recording electrode was amplified, filtered (bandpass, 200–2000 Hz) and displayed on a Tetronix oscilloscope, using a WPI-121 window discriminator to isolate single cell activity. A signal-to-noise ratio of 2:1 was the minimum requirement for discriminating a cell. The single-cell activity was recorded by a spike-frequency counting program on a Commodore computer. Cells were monitored for two 120 s trials, where the firing rates of 10 s bins were averaged to determine the mean firing rate of the unit. On average, four to six cells were sampled per hour, with a recording time of 8–12 h providing a total of forty to seventy units of data with which to assess the SCN electrical activity rhythm for each peak studied. Neuronal activities were sampled at random throughout the SCN. Previous studies have shown that the SCN functions as a coherent pacemaker with a uniform rhythm of electrical

activity measurable in both dorsomedial and ventrolateral regions (Tcheng, Gillette & Prosser, 1989). The recorded firing rates of individual cells were averaged together and 2 h means, with 15 min lags, were calculated and plotted. The time-of-peak, defined as the circadian time at which the 2 h mean of the firing rates of the sampled cells reached a symmetrical maximum, was determined. The experimental time-of-peak was compared to microdrop controls in order to determine the magnitude of the phase shift induced by the experimental treatments.

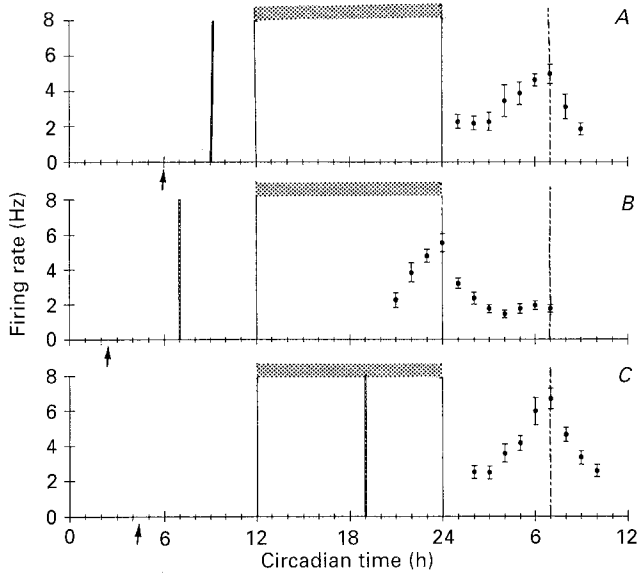

Fig. 2. The SCN sensitivity to phase shifting by serotonin changes over the course of the day. *A*, slices treated with a microdrop of medium at CT 9 on day 1. Rhythm of endogenous neuronal activity on day 2 peaked at CT 6·9. *B*, slice treated with a microdrop of 10^{-6} M-serotonin at CT 7 on day 1. The peak in the rhythm of electrical activity on day 2 was phase advanced 7 h to CT 0. *C*, slice treated at CT 19, on day 1. The peak occurred at CT 7 on day 2 which is overlapping with the peak in the rhythm of control slices. ● represent the 2 h means ± S.E.M., plotted with 1 h lags, of the recorded neuronal activity rhythm on the second day. The vertical bar indicates the time of serotonin treatment and the interrupted line indicates the time-of-peak observed in untreated slices. The horizontal stippled bar indicates the time of the donor's subjective night in the colony. Arrows point out the time of slice preparation.

RESULTS

Control experiments

Because the time-of-peak of electrical activity in the SCN is highly predictable between experiments and stable over time *in vitro* (CT 6·9 ± 0·2, $n = 8$, Prosser & Gillette, 1989), as well as easy to visually discern, we use it to mark the phase of the underlying circadian pacemaker. In the present series of experiments, the time-of-peak for untreated SCN of rats from our inbred colony occurred at CT 6·9 ± 0·1 ($n = 3$), which is in agreement with previously reported results. In the microdrop controls, applied at CT 9, the time-of-peak was at CT 6·9 ± 0·1 ($n = 3$, Fig. 2*A*), which is identical to the established peak times for untreated SCN. This demonstrates that the microdrop technique did not, in itself, cause phase shifts.

Serotonin experiments

We found that serotonin can affect the SCN pacemaker. Single-unit recordings of the population of neurons revealed that when a microdrop of serotonin was administered in mid-subjective day on the first day *in vitro*, the peak of the next

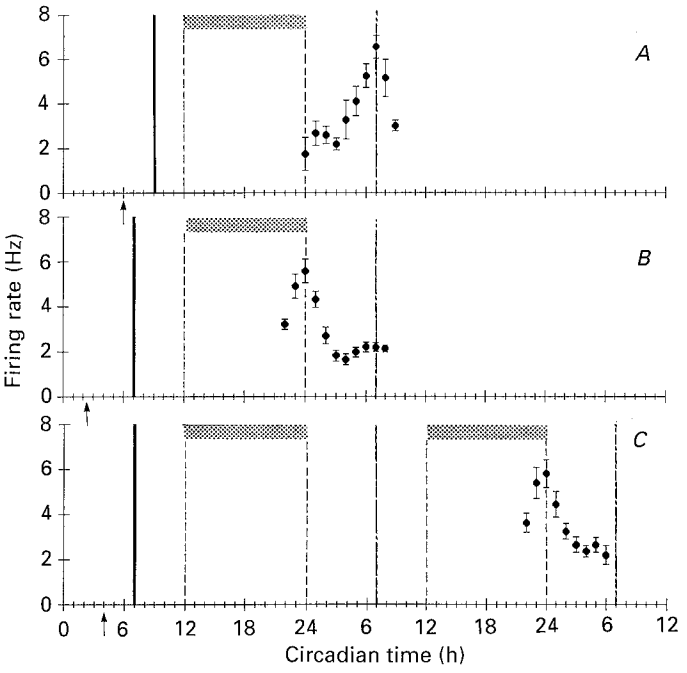

Fig. 3. Serotonin induced permanent phase shifts *in vitro*. *A*, rhythm of endogenous neuronal activity recorded on day 2 after treatment with medium on day 1. *B*, localized application of serotonin to the ventrolateral region of the SCN slice at CT 7 resulted in a 7·0 h phase advance in the rhythm of electrical activity on day 2. *C*, recording on day 3, in a separate experiment, following treatment with serotonin at CT 7 on day 1, indicated a 7·0 h phase advance. This is overlapping with the mean phase advance seen on day 2. ● represent the 2 h means ± S.E.M. of the recorded neuronal activity rhythm on the second and third day. The vertical bar indicates the time of serotonin treatment and the interrupted line indicates the time-of-peak observed in control slices. The horizontal stippled bar indicates the time of the donor's night in the colony. Arrows point out the time of slice preparation.

day's rhythm of electrical activity was significantly advanced. After a 5 min application of serotonin at CT 7 on day 1, the time-of-peak occurred at CT 23·9 ± 0·1 ($n = 3$) on day 1, rather than at CT 6·9 ± 0·1, the time-of-peak on day 2 in control slices (Fig. 2*B*). This is a 7·0 ± 0·1 h advance in the neuronal activity rhythm. Statistical analysis of the data using Student's t test, which compared the time-of-peak in serotonin-treated slices to that of control confirms that the effect of serotonin at CT 7 is significant ($P < 0·001$). In a separate experiment, the electrical activity rhythm was recorded on day 3 after serotonin treatment on day 1 at CT 7 (Fig. 3). The peak appeared at CT 0, 23·9 h after the peak on day 2, and still advanced in

phase by ~ 7 h. These results indicate that the phase change due to serotonin treatment at CT 7 on day 1 is a permanent one.

Administration of serotonin in the subjective night, however, had no apparent effect on the rhythm of neuronal activity on the second day. After exposure of the

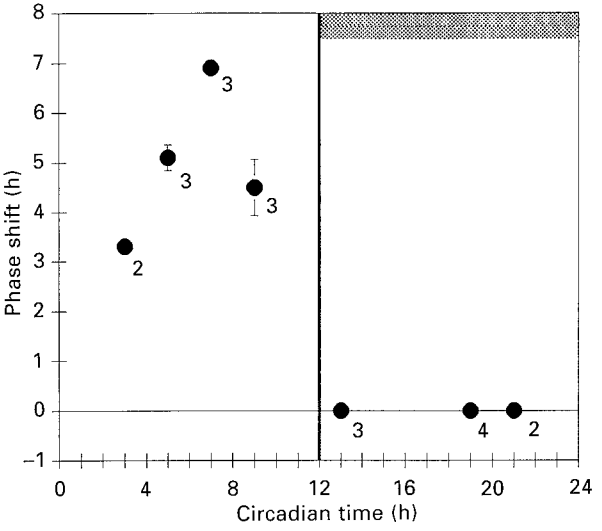

Fig. 4 Phase-response curve for serotonin. The x-axis denotes circadian time (CT) of treatment (h) and the y-axis indicates the average magnitude and direction of the serotonin induced phase shift (h). The magnitude of the shift of the time-of-peak of the electrical activity rhythm was determined in relation to time-of-peak in slices treated with medium. ● represent the mean ± S.E.M. phase shift. The subscript number is the number of experiments performed at a particular time point. The vertical bar denotes the time of 'lights off' in the colony and the horizontal stippled bar indicates the night.

SCN to serotonin at CT 19, the peak in activity on the next day was at CT 6·7 ± 0·1 ($n = 3$, Fig. 2*C*). This time overlaps with controls.

Similar measurements were made after a microdrop of serotonin was applied at one of three other time points during the subjective day, CT 3 ($n = 2$), 5 ($n = 3$), 9 ($n = 3$), and two other points during the subjective night, CT 13 ($n = 3$), and 21 ($n = 2$). A phase-response curve relating the circadian time of serotonin treatment to its effect on the phase of the electrical activity rhythm is seen in Fig. 4. Treatment with serotonin in the subjective day-time resulted in robust, persistent phase advances in the rhythm of neuronal activity, while it had no effect on the pacemaker in the subjective night.

Agonist experiments

5-Carboxamidotryptamine

Treatment of slices with 5-carboxamidotryptamine, a serotonergic agonist specific for the 5-HT_1 receptor subtype (Peroutka, 1988), resulted in time-dependent phase shifts in the rhythm of neuronal activity. When administered during the day at CT 9, a time when serotonin induced a 4·6 h phase advance, 5-carboxamidotryptamine

caused a 6·0±0·1 phase advance ($n = 3$, Fig. 5*A*). Conversely, treatment of SCN at CT 15 had little effect on the phase of the rhythm of electrical activity (Fig. 5*C*). The time-of-peak was recorded at CT 6·5 ($n = 2$) which is near the control peak time.

8-OH DPAT

Treatment at CT 9 with 8-OH DPAT, an agonist specific for the 5-HT_{1A} receptor subtype (Middlemiss & Fozard, 1983), also resulted in significant phase shifts in the

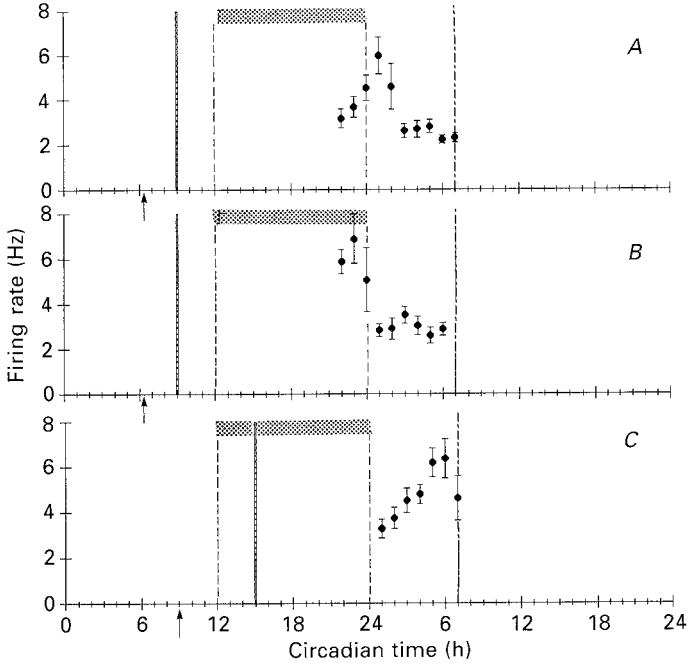

Fig. 5. Serotonergic agonists phase-shift the rhythm of electrical activity. *A*, slice treated at CT 9 on day 1 with 5-carboxamidotryptamine. The time-of-peak on day 2 occurred 6·5 h earlier than in control slices. *B*, slice treated at CT 9 on day 1 with 8-OH DPAT. The peak in the rhythm of electrical activity, recorded on day 2 was advanced by 8 h. *C*, slice treated on day 1 with 5-carboxamidotryptamine at CT 15. The time-of-peak was recorded at CT 6·25 which is overlapping with the range of peak times in control slices. ● represent the 2 h means±S.E.M. of the recorded neuronal activity rhythm on the second and third day. The vertical bar indicates the time of serotonin treatment and the interrupted line indicates the time-of-peak observed in slices treated with medium. The horizontal stippled bar indicates the time of the donor's night in the colony. Arrows point out the time of slice preparation.

rhythm of neuronal activity. The time-of-peak was observed at CT 0·0±0·1 ($n = 3$), indicating a 7 h phase advance (Fig. 5*B*).

DISCUSSION

Our results on the suprachiasmatic slice preparation demonstrate that serotonin can act directly on the SCN to change the phase of the pacemaker. The responses at the time points tested indicate that SCN sensitivity to serotonin released at raphe

projection sites is limited to the subjective day of the circadian cycle. Serotonin treatment on day 1 *in vitro* phase advances the rhythm of neuronal activity within the next 12 h so that the peak appears early on the second and third day. The stable phase relationship between days 2 and 3 suggests that the pacemaker has been permanently reset by the brief (5 min) exposure to serotonin. The changes in the phase of the pacemaker were also shown to be serotonin specific, as demonstrated by the agonist-induced phase shifts.

Analysis of SCN in the brain slice, where it is isolated from modulating inputs and feedback loops from other brain regions, permits direct assessment of properties intrinsic to the pacemaker. This approach therefore provides insight to the basic characteristics of the system. The present study suggests that serotonin can induce 7 h phase changes in the pacemaker, should the other modulatory pathways be absent. While such large phase shifts are not uncommonly observed in the slice preparation (cyclic AMP induces 5 h phase shifts, Prosser & Gillette, 1989; K^+ induces 6 h phase shifts, Gillette, 1987), shifts of this magnitude have rarely been reported *in vivo*, where the magnitude of the effect of a 1 h stimulus does not exceed 2 h. In addition to the difference in magnitude, phase shifts *in vitro* differ in rate. We have not observed transient changes in the phase of the rhythm of electrical activity over successive days *in vitro* (Gillette, 1987). Comparison of data sets indicates that the pacemaker can be reset directly and rapidly *in vitro*, but that *in vivo* it is modified by numerous modulatory influences and feedback loops such that the phase shifts are damped in magnitude and rate (as in DeCoursey, 1964). From the massive size of the serotonin phase advance relative to day-acting zeitgebers *in vivo* it follows that potent inertial forces normally act to slow the size and rate of the change. However, the fact that this is one of the largest phase advances that we have found among agents studied *in vitro* may also be a reflection of the strength of this pathway relative to other modulatory forces acting in the same time domain.

While the effect of serotonin injection into the SCN *in vivo* has not yet been examined, indirect evidence suggests that serotonergic pathways may stimulate phase shifts in behaviour in the animal during the day-time. Forced activity in the middle of the day facilitates entrainment of mammals to novel light–dark schedules (Mrosovsky & Salmon, 1987). Hamsters that were confined to a running wheel after being exposed to a phase-altered lighting schedule re-entrained more than twice as rapidly as undisturbed animals. Further experiments indicated that hamsters undergo accelerated phase shifting by behavioural arousal, by forced activity or social interaction at midday (Mrosovsky, 1988), a time when serotonin induces phase shifts *in vitro*. The physiological mechanism by which non-photic signals entrain the SCN has not yet been determined. Mrosovsky (1988) has hypothesized that state of arousal is communicated to the SCN via serotonergic projections from the raphe nuclei. Our data lend support to this notion.

Interestingly, the SCN period of sensitivity to serotonin overlaps the period of sensitivity to cyclic AMP. Application of cyclic AMP analogues resulted in phase advances in the rhythm when applied in the subjective day (CT 3–11) with a peak sensitivity between CT 5 and 7; treatment in the subjective night did not significantly affect the rhythm of electrical activity (Gillette & Prosser, 1988). Similarly, administration of substances that elevate the endogenous levels of cyclic AMP

(phosphodiesterase inhibitors that prevent cyclic AMP breakdown or stimulators of adenylate cyclase) altered the phase of the rhythm of neuronal activity in a similar manner (Prosser & Gillette, 1989).

Extensive research on the organization of the endogenous pacemaker in the eye of the mollusc *Aplysia* has led to the conclusion that serotonin plays a regulatory role in that circadian pacemaker. *In vitro* experiments with the *Aplysia* ocular pacemaker (Corrent, Eskin & Kay, 1982) have demonstrated that serotonin acts as a strong phase-shifting agent through a sequence of events separate from the light entrainment pathway. Bath applications of serotonin to preparations of the isolated eyes of *Aplysia*, for a minimum pulse of 1·5 h, resulted in phase advances of 3–4 h between CT 5 and 11 and phase delays of 2·5 h from CT 22 to CT 2 (Corrent, Mcadoo & Eskin, 1978).

Phase shifts similar to those induced by serotonin were stimulated by cyclic AMP and its analogues (8-benzylthio-cyclic AMP) in the *Aplysia* pacemaker (Eskin, Corrent, Lin & Mcadoo, 1982). In addition, administration of phosphodiesterase inhibitors (3-isobutyl-1-methylxanthine, IBMX, RO20-1724 and papaverine) that block cyclic AMP breakdown resulted in serotonin-like phase-shifted rhythms (Eskin *et al.* 1982). When the phosphodiesterase inhibitor was administered simultaneously with serotonin, no augmentation was seen suggesting that cyclic AMP and serotonin were acting through the same pathway (Eskin *et al.* 1982). Additional support for this proposition comes from the finding that serotonin produces changes in the endogenous levels of cyclic AMP in the eye of *Aplysia* (Eskin *et al.* 1982). Further steps in the serotonin pathway have also been studied. The protein synthesis inhibitor, anisomycin, blocks serotonin phase-shifting after the cyclic AMP step suggesting a requirement for synthesis of a specific protein or increased levels of certain proteins for the cyclic AMP effect (Eskin, Yeung & Klass, 1984; Yeung & Eskin, 1987).

Comparison of our results in rat SCN with the sensitive circadian periods in the *Aplysia* ocular pacemaker reveal a surprising correlation. The sensitive period of rat SCN to phase advance by serotonin is CT 3–9; that of *Aplysia* eye is CT 5–11. This temporal correlation of circadian pacemaker sensitivity to serotonin- and cyclic AMP-induced phase shifts in such phylogenetically distant organisms suggests conservation of circadian pacemaker mechanisms. The maximum phase shifts observed in the rat ($7{\cdot}0 \pm 0{\cdot}1$ h) were significantly greater than the 3·5 h phase shift in *Aplysia* (Eskin *et al.* 1982). The treatment times that we used were also more than an order of a magnitude shorter, 5 min *vs.* 1·5 h. The mechanism underlying these differences in sensitivity is unclear, but such differences are characteristic of the respective sensitivities of these two organisms to other circadian phase-shifting stimuli.

The phase shifts observed after treatment with serotonergic agonists suggest that a 5-HT_1 receptor may be involved in mediating serotonergic signals to the SCN. Neither definitive classification, nor functional and anatomical distribution of the serotonergic receptor subtypes present in the SCN has been determined. The 5-HT_1 receptors comprise a family of receptor subtypes that are radiolabelled with [^{3}H]5-HT. The 5-HT_{1A} receptors have been identified by radiolabelling with [^{3}H]8-OH DPAT (Middlemiss & Fozard, 1983; De Vivo & Maayani, 1986). They are a distinct

group of receptors that work through an adenylate cyclase pathway in a number of systems. There is conflicting evidence in terms of the mechanism of action of the 5-HT_{1A} receptors, as there are some reports that indicate that it acts through elevation of cyclic AMP levels (Markstein, Hoyer & Engel, 1986), while others suggest that it is negatively coupled to cyclic AMP (Weiss, Sebben, Kemp & Bockaert, 1986). Our results not only confirm the specificity of the serotonin-induced phase shifts but also lend support to the notion of a possible cyclic AMP-coupled mechanism.

The effects of quipazine, a non-specific serotonergic agent with reported agonistic and antagonistic effects (Peroutka, 1988), have been tested with bath application to SCN-containing hypothalamic slices (Prosser, Miller & Heller, 1990). Quipazine induced more modest (4 h) phase advances of SCN rhythms in the day-time compared with serotonin; treatment at night induced 4 h phase delays. That study differed both pharmacologically and methodologically from ours in ways that would contribute to the differing results. It is surprising that the hour-long exposure of the whole slice to a higher concentration of quipazine should produce significantly lower amplitude phase advances than a very localized, brief application of a lower concentration of serotonin. However, an extensive range of serotonin receptor sites and subtypes have been reported throughout the hypothalamic region included in the slice (Dean, Miller & Dement, 1990) and all would be exposed to quipazine during bath application.

Together these two studies suggest that there may be topographic variations in the function of serotonin receptors in the SCN and nearby hypothalamus. In the day-time quipazine may have produced different effects at the various serotonin receptor sites and types that summed to damp the full shift inducible at dorsal raphe projection sites. More intriguing is the phase delay produced at night by quipazine, but not serotonin or 5-carboxamidotryptamine. It cannot be explained by instability of the serotonin solution: serotonin was applied within 15 min of preparation and 5-carboxamidotryptamine, a stable analogue, produced the same responses. The finding that phase delays were stimulated only with bath application suggests that perhaps phase delays and phase advances are mediated through different pathways in the mammalian circadian system. Serotonergic pathways that terminate at hypothalamic regions near the SCN, but do not overlap with the retino-hypothalamic, geniculo-hypothalamic or raphe projections to the ventrolateral SCN would be candidates. This provocative hypothesis deserves further investigation.

Our study demonstrates a regulatory role for serotonin in the rat SCN. While the raphe input is not necessary to sustain circadian rhythmicity *in vitro*, it may play an integrative role in the temporal organization of the mammalian circadian system. The raphe acts to modulate arousal states and integrate behaviour in response to changes in motor activity, wakefulness and other physiological functions. In the circadian system serotonin from the raphe may serve as a messenger of daily, rhythmic changes in the arousal state of the animal. Stimulation of the serotonergic system alters the phase of the pacemaker *in vitro*, and it may function similarly *in vivo* to synchronize the pacemaker to social or behavioural changes in the environment.

This study was supported by the US Air Force Office of Scientific Research (90-0205). Preliminary findings were presented to the Society for Neuroscience, 1990.

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