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Research report

Serotonin phase-shifts the mouse suprachiasmatic circadian clock in vitro

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Abstract

The mammalian circadian clock in the suprachiasmatic nucleus (SCN) receives multiple afferent signals that could potentially modulate its phase. One input, the serotonin (5-HT) projection from the raphe nuclei, has been extensively investigated in rats and hamsters, yet its role(s) in modulating circadian clock phase remains controversial. To expand our investigation of 5-HT modulation of the SCN clock, we investigated the phase-shifting effects of 5-HT and its agonist, (+)8-hydroxy-2-(di-*n*-propylamino)tetralin (DPAT), when applied to mouse SCN brain slices. 5-HT induced 2–3 h phase advances when applied during subjective day, while non-significant phase shifts were seen after 5-HT application at other times. These phase shifts were completely blocked by the 5-HT antagonist, metergoline. DPAT also induced phase shifts when applied during mid-subjective day, and this effect appeared dose-dependent. Together, these results demonstrate that the mouse SCN, like that of the rat, is directly sensitive to in vitro phase-resetting by 5-HT.

Theme: Autonomic, limbic and other systems

Topic: Biological rhythms and sleep

Keywords: Circadian rhythm; Suprachiasmatic nucleus; Serotonin; Brain slice; Phase-shift; Mouse

1. Introduction

The circadian pacemaker located in the suprachiasmatic nucleus (SCN) controls daily behavioral and physiological rhythms in mammals [15]. The ability of the SCN pacemaker to continue generating circadian rhythms when isolated in a brain slice preparation [10] or as dispersed cells [50] underscores its endogenous nature.

The mammalian circadian clock maintains synchrony with the external environment primarily through photic information relayed from the retina through the glutamatergic retinohypothalamic tract (RHT) to the SCN [27,29]. Light or glutamate can delay or advance the circadian clock depending upon whether it is presented during the early or late night, respectively [45]. In contrast, 'nonphotic' stimuli such as timed access to a running wheel and sleep deprivation have been shown to phase-shift the circadian clock when presented during the daytime [13,24,34]. Because the SCN receives a substantial serotonergic (5-HTergic) projection [28,32] and 5-HT activity is generally correlated with increased activity/arousal [6,7,23,46,47], many studies have investigated the possibility that 5-HT participates in these nonphotic phase shifts (for review see Refs. [13,26,30]).

The results of these studies are mixed, which has led to much confusion concerning the role of 5-HT in modulating circadian clock phase. Some of the discrepancies may be due to species and strain differences, and/or they may be due to the use of in vivo vs. in vitro experimental paradigms. For example, 5-HT and its agonists have generally induced robust nonphotic-like phase shifts when applied to rat brain slices, while inconsistent effects are seen when administered to hamsters in vivo (for review, see Refs. [17,26]).

Very few studies have investigated the phase-shifting effects of 5-HT in the mouse. Only one study thus far has specifically investigated whether 5-HT agonists can induce

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phase shifts in vivo, and it found that systemic injections of the 5-HT agonist, (+)8-hydroxy-2-(di-*n*-propylamino)tetralin [(+)DPAT], do not induce phase shifts in mice (Antle et al., in press). However, results from several studies suggest that the 5-HT afferents to the SCN are involved in nonphotic phase shifts in mice. Intra-SCN injections of the neurotoxin, 5,7-dihydroxytryptamine (5,7-DHT), prevent entrainment of mice to either a treadmill [21] or access to a running wheel [8] without changing the amount of activity expressed [8]. 5,7-DHT injections also increase the free-running period and the duration of activity, and advance the time of peak activity [31].

In contrast, substantial research demonstrates that 5-HT can act in the mouse SCN to inhibit photic phase shifts. This effect appears to involve both 5-HT_{1B} receptor inhibition of glutamate release [3,37,38,48] as well as 5-HT_7 receptor inhibition of postsynaptic glutamate responses [3,48].

To further clarify the extent to which 5-HT can modulate mouse SCN circadian clock activity, I investigated whether in vitro application of 5-HT and the 5-HT agonist, (+)DPAT, can phase-shift the mouse circadian clock. Having identified an in vitro effect, I further characterized its phase- and dose-dependency.

2. Materials and methods

2.1. Brain slice preparation

Coronal brain slices (500 μm) containing the SCN were prepared during the daytime from adult, male C57BL/J6 mice, housed in 14:10 LD conditions as reported previously [39,43,44]. Slices to be used in 1–2 day experiments were all prepared between Zeitgeber time (ZT) 0–4 (where ZT 12=lights-off in the donor animal colony), while those used in 3 day experiments were prepared slightly later in the day (ZT 7–7.5). Slices were maintained at the interface of a Hatton-style brain slice chamber [14], where they were perfused continuously with warm (37 °C), oxygenated (95% O_2/5% CO_2), glucose/bicarbonate-supplemented Earle's balanced salt solution (EBSS; Sigma), pH 7.4–7.5. Gentamicin (0.05%) was also added to the perfusion medium.

2.2. Experimental protocols

2.2.1. Drug treatment

At the specified time, perfusion through the slice chamber was stopped and the medium in the slice chamber was replaced with normal medium or medium supplemented with 5-HT (10 μM), or the 5-$HT_{1A/7}$ agonist, (+)DPAT (10 μM). After 10 min this medium was replaced with the normal medium and perfusion was reinstated.

2.2.2. Blocking experiments

Beginning 5 min prior to ZT 6, perfusion was stopped and the medium in the chamber replaced with medium containing the nonselective 5-HT antagonist, metergoline (10 μM). At ZT 6 this medium was switched to one containing both metergoline and 5-HT. After 10 min slices received a final 5 min treatment with medium containing metergoline, after which the normal medium was re-introduced to the slice chamber and perfusion reinstated.

2.3. Single unit recordings and data analysis

Single unit (SUA) recordings generally commenced early on day 2 in vitro. For the majority of experiments this was 18–24 h after the end of drug treatment and slightly more than 24 h after slices were prepared. However, for those experiments where this would have necessitated recording immediately following treatment (i.e., 5-HT applied at ZT 24), SUA recordings were delayed to the third day in vitro, commencing approximately 23 h after drug treatment. This time interval between drug treatment and SUA recording is consistent with that of the majority of experiments presented here, and was done to ensure that the observed pattern of neuronal activity accurately reflected the phase of the underlying pacemaker rather than being influenced by acute actions of 5-HT on membrane potential. The procedure for SUA recordings has been described previously [39,44]. Briefly, the spontaneous activity of single SCN neurons was recorded using glass capillary microelectrodes filled with 3 M NaCl. Each neuron was recorded for 5 min, and the data stored for later determination of firing rate using a Data*Wave* system (Longmont, CO). Typically, 4–7 cells were recorded during each hour. These individual firing rates were then used to calculate 2 h running averages (composed of 8–14 individual firing rates), lagged by 1 h (±S.E.M.), to obtain a measure of population neuronal activity. As in previous studies [39,44], the time of peak neuronal activity was assessed visually by estimating, to the nearest quarter hour, the time of symmetrically highest activity. Phase shifts were calculated as the difference in time-of-peak of untreated slices vs. drug-treated slices. Using these methods, the consistency of the results obtained for each experimental manipulation is such that differences in phase of slightly more than 1 h are often statistically significant with few (n=2 to 3) replicates (e.g., Refs. [39–41]).

2.4. Statistical analysis

Differences in time of peak neuronal activity were assessed using Student's t-test. In all cases, the level of significance was set at $P<0.05$.

3. Results

3.1. In vitro mouse SCN neuronal activity rhythms

Neuronal activity recordings from untreated mouse SCN slices revealed a robust rhythm in neuronal activity with a peak near mid-subjective day on both the first and second days in vitro (Fig. 1A, B). The mean time of peak activity in untreated slices was ZT 3.92±0.1 h (n=3) on day 1 and ZT 4.25±0.2 h (n=5) on day 2. In one series of experiments brain slices were treated for 10 min at ZT 6 with the normal perfusion medium (EBSS) following the same protocol subsequently used in drug treatment experiments.

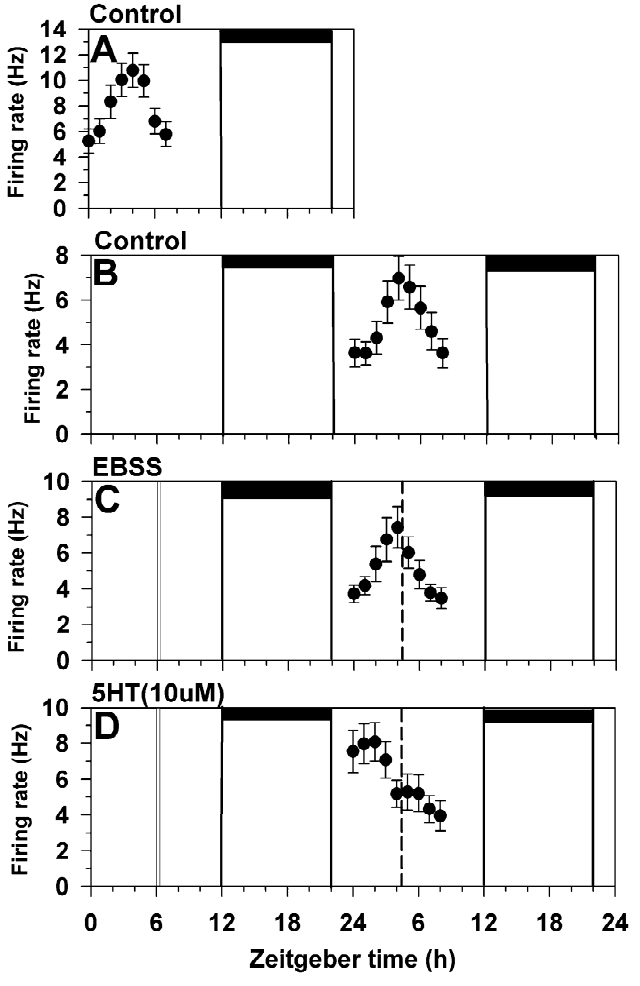

Fig. 1. Serotonin phase-shifts the mouse circadian clock in vitro. Shown are the 2 h means±S.E.M. of SCN neuronal activity obtained (A) in a control experiment on day 1 in vitro; (B) in a control experiment on day 2 in vitro; (C) after treatment with the normal perfusion medium; and (D) after treatment with 10 μM 5-HT. Neuronal activity peaked near ZT 4 in both control experiments and after EBSS treatment, while the peak in neuronal activity occurred near ZT 1.5 after 5-HT treatment. Thus, 5-HT induced a 2.75 h phase advance in the neuronal activity rhythm. Horizontal bars: time of lights-off in the animal colony; vertical bar: time of drug treatment; dotted line: mean time-of-peak in control experiments.

Treating the slices with EBSS alone did not perturb the pattern of neuronal activity (Fig. 1C), and the mean time of peak activity was not altered (mean time-of-peak=ZT 4.0±0.0, n=3; P>0.05 vs. untreated slices). Therefore, in subsequent experiments the mean time of peak neuronal activity in treated slices was compared to that of untreated slices.

3.2. Serotonin phase-advances the mouse SCN pacemaker in vitro

Mouse SCN brain slices treated for 10 min with 5-HT(10uM) at ZT 6 exhibited an advanced rhythm in neuronal activity (Fig. 1D). The mean time of peak activity in these slices was ZT 1.5±0.2 (n=3), corresponding to a 2.75 h advance. This time of peak activity was significantly different from that of controls (P<0.05).

3.3. Metergoline blocks 5HT-induced phase advances in vitro

To begin assessing whether 5-HT was phase-shifting the clock specifically through stimulating 5-HT receptors, I investigated the effects of the nonselective 5-HT antagonist, metergoline. When applied alone at ZT 6, metergoline (10 μM) did not alter the time of peak activity (mean time-of-peak=ZT 4.25±0.4, n=3). However, when co-applied with 5-HT, metergoline completely blocked the 5-HT-induced advance in neuronal activity (mean time-of-peak=ZT 4.0±0.2, n=3; Fig. 2B).

3.4. (+)DPAT induces phase advances in vitro

To further define the nature of the 5-HT-induced phase shifts, I investigated the effects of the 5-$HT_{1A,7}$ agonist, (+)DPAT on mouse SCN neuronal activity rhythms. A 10 min application of (+)DPAT (10 μM) to mouse SCN brain slices at ZT 6 induced a phase advance similar in magnitude to that induced by 5-HT (mean time-of-peak=ZT 1.65±0.2, n=5; Fig. 2A). This 2.6 h advance represents a significant change from controls (P<0.05). The phase-shifting effect of (+)DPAT appears to be dose-dependent (Fig. 3), with an ED_{50} of approximately 0.05 μM.

3.5. Phase-dependency of serotonergic phase shifts in vitro

Finally, I investigated whether the phase shifts induced by 5-HT depend on the time of treatment. 5-HT (10 μM) was applied for 10 min to mouse SCN slices at different times across the circadian cycle, and the time of peak neuronal activity was assessed the following day. As shown in Fig. 4, 5-HT induced phase advances during mid-subjective day (ZT 2–8), with the largest advances seen after 5-HT application at ZT 6. No consistent effects

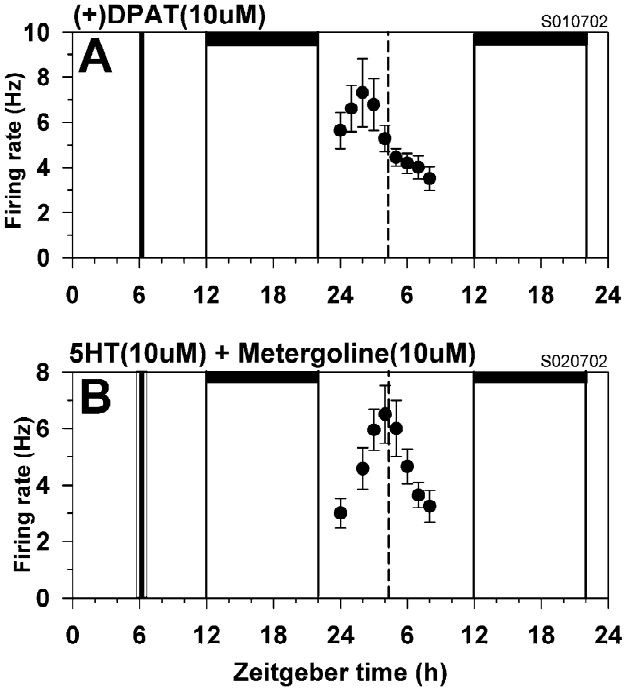

Fig. 2. Serotonin-induced phase shifts are mimicked by (+)DPAT and blocked by metergoline. Shown are the 2 h means±S.E.M. of SCN neuronal activity obtained (A) after 10 min treatment with 10 μM (+)DPAT at ZT 6; and (B) after co-treatment with 5-HT (10 μM) and metergoline (10 μM). (+)DPAT induced a phase advance of about 2.25 h, while metergoline completely blocked the phase advance normally induced by 5-HT. See Fig. 1 legend for details.

of 5-HT were seen in response to 5-HT application at other times, although small, nonsignificant delays were observed after 5-HT application at ZT 22–24.

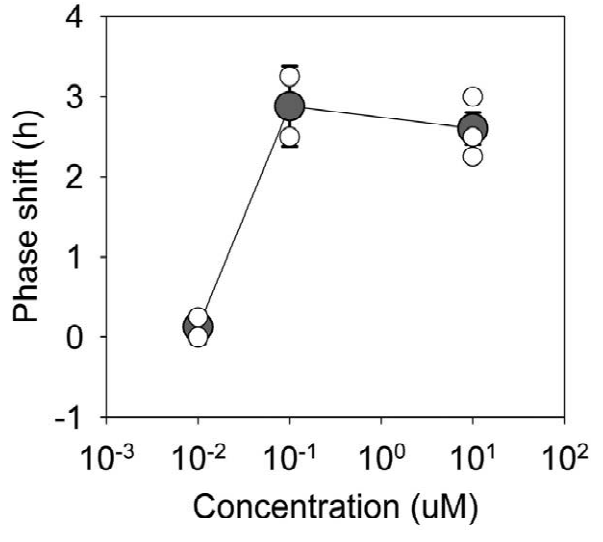

Fig. 3. Dose-dependence of serotonergic phase shifts. Shown are the mean phase advances (±S.E.M.) induced by (+)DPAT application (grey circles) and the phase advances induced in individual experiments (open circles). Maximum phase advances are induced by (+)DPAT concentrations down to 0.1 μM, while no shift is seen with 0.01 μM (+)DPAT.

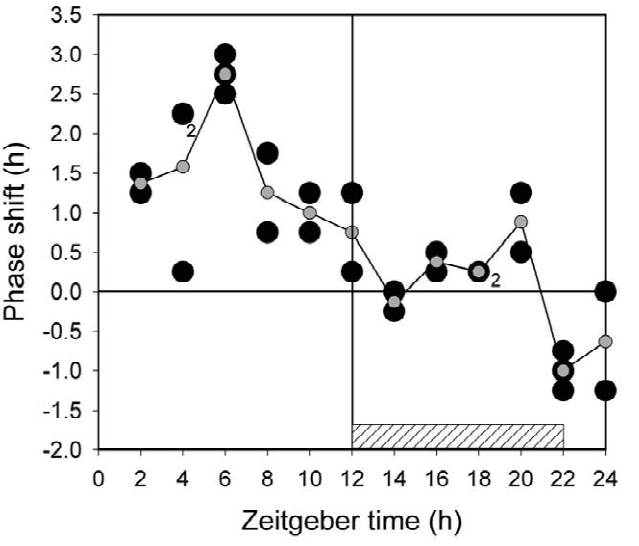

Fig. 4. Phase response curve for 5-HT. Shown are the shifts in time-of-peak seen in individual experiments (black circles) after 10 min 5-HT (10 μM) treatment at various times throughout the circadian cycle, along with the means (grey circles) for each phase tested. The largest phase shifts are seen after 5-HT application at ZT 6.

4. Discussion

The results of these experiments clearly demonstrate that the mouse SCN circadian pacemaker can be phase-shifted by 5-HT acting on receptors contained within the SCN. This is the first definitive evidence that the mouse SCN is directly sensitive to phase-resetting by 5-HT.

The times of peak activity recorded in these experiments on day 1 and day 2 indicate the circadian clock is running with an in vitro period near 24 h. This is consistent with the mean free-running period of about 23.5 h seen in vivo [25,49] as well as the near-24 h rhythms recorded from cultured mouse SCN slices [12,18,51] and dispersed cells [16,35] (however, see Ref. [2]). While previous SUA recordings from mouse SCN slices have not reported an in vitro period, the time-of-peak on day 2 in those studies was around ZT 6–7 [1,19], which is consistent with our data in that this corresponds to 6 h after projected lights-on. It is also similar to our in vitro recordings from mice previously housed under 12:12 LD conditions (unpublished results). Using this estimated 24 h in vitro rhythm in SUA seen between days 1 and 2, we can project that the unperturbed time of peak likely would occur at approximately the same time on subsequent days.

The 5-HT-induced phase shifts seen in these experiments are similar to activity/arousal-induced phase shifts observed in mice [5,20,22], in that the largest phase shifts occur in response to stimuli applied during the mid-subjective day, while smaller phase delays are sometimes seen near subjective dawn. This similarity in the pattern of phase shifts seen in vivo and in vitro may not be coincidental. Intense arousal leads to increased activity of

raphe neurons, and subsequently, increased 5-HT release in the SCN in other animals [6,7,23,46]. Furthermore, data from Marchant et al. [21] and Edgar et al. [8] suggest that in mice, entrainment to daily exercise requires intact 5-HT afferents to the SCN. Because these studies used 5,7-DHT injections into the SCN to eliminate 5-HT afferents, it is important to note that the injections could have also damaged 5-HT projections to other regions [30]. Nevertheless, the data are consistent with a modulatory role for 5-HT in the SCN circadian clock.

Despite the clear evidence shown here that 5-HT can directly phase-shift the mouse circadian pacemaker, comparable data from in vivo experiments, with intra-SCN injections of 5-HT agonists, is lacking. In fact, a recent study shows that systemic injections of DPAT in mice do not induce daytime phase advances (Antle et al., in press). While the results of that study are not directly comparable to those presented here, since the DPAT was not applied directly to the SCN in both cases, this nevertheless suggests the mouse SCN clock may respond differently to in vivo vs. in vitro 5-HT. There are several possible explanations for this potential change in responsiveness. One is that the process of isolating brain tissue in vitro increases the extracellular space surrounding the neurons, thereby increasing the ability of exogenous chemicals to stimulate receptors confined to the narrow synaptic clefts [36]. If this is the case, then one might speculate that intra-SCN injections of DPAT would induce phase shifts in mice, but that a higher concentration of the agonist might be needed.

Another possibility is that 5-HT receptor insensitivity limits in vivo serotonergic phase-shifts. It was recently shown that DPAT injections into the SCN induce larger phase-shifts after pre-treating hamsters with the reversible 5-HT toxin, *p*-chlorophenylalanine (PCPA) [9]. By limiting serotonergic activity prior to the phase-shifting treatment, PCPA may have increased 5-HT receptor sensitivity in the SCN. A similar increase in receptor sensitivity might occur in vitro due to the cessation of spontaneous 5-HT release in the de-afferented SCN.

A third possible explanation is that non5-HT afferent stimuli inhibit serotonergic phase shifts in vivo. 5-HT-induced phase shifts in vitro are blocked by a variety of stimuli, including NPY, melatonin, and Glu [39,41,42]. The fact that light pulses inhibit activity/arousal-induced phase advances [4,11,33] and 5-HT-induced phase shifts in hamsters [9] adds credence to the theory that inhibitory interactions occur between SCN afferents, and that these afferent inputs could be inhibiting in vivo serotonergic phase shifts.

Should differences between in vivo and in vitro phase-shifting by 5-HT be clearly demonstrated, one might be tempted to dismiss the in vitro data as artifact and not pursue it further. However, the in vitro data clearly provide an insight into those signals to which the clock is capable of responding, and therefore they tell us something about the clock. Our goal, therefore, must be to determine which factors dictate the sensitivity of the circadian clock to phase-shifting stimuli.

While few studies have investigated 5-HT-induced phase shifts in mice, several studies have shown that 5-HT inhibits photic phase shifts in mice. The 5-HT_{1B} agonist, TFMPP blocks photic phase shifts, c-fos induction, and optic nerve stimulation-induced, but not glutamate-induced, postsynaptic currents [37,38,48]. Together with histological evidence [3], these data indicate 5-HT_{1B} receptors are located on retinal terminals and act by inhibiting photic-induced glutamate release in the SCN. Additional data suggest that 5-HT_7 receptors located postsynaptically in the SCN may further suppress the effects of light on the circadian clock [3,38].

In conclusion, the data presented here clearly show direct 5-HT modulation of the mouse circadian clock under in vitro conditions. Together with previous data, therefore, our results indicate that 5-HT may play two distinct roles in the mouse SCN: it can inhibit photic phase shifts at night and it can independently phase-shift the circadian clock during the day. It will be important in subsequent research to determine the conditions that influence when these effects occur.

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