Synchronization and entrainment of the suprachiasmatic nucleus by phase-dependent responses of coupled clock cells to GABAergic inputs

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The hypothalamic suprachiasmatic nucleus (SCN), the biological master clock in mammals [1], contains thousands of autonomous, circadian oscillators (i. e., clock cells) having different intrinsic periods (20 $\sim$ 28 hr) [2] Yet, these cells *in situ* can synchronize themselves to generate a coherent rhythm and be entrained to external, cyclic environments. For the SCN to exhibit synchronization and phase-resetting responses, cell-to-cell interactions should exist, and a promising agent mediating the interactions is the neurotransmitter *γ-aminobutyric acid* (GABA) [3–11] So far, however, there has been no explicit demonstration that GABA has an essential role for the SCN to work as a functional unit. Besides, the precise mode of GABA action in clock cell synchronization is unknown. Here, we show that $GABA_A$ receptor-mediated cell-to-cell interaction, operating at slow time scales ($>$ 10 sec), is essential for the population of SCN cells to generate a coherent rhythm. Following these observations, we have developed a novel model of the SCN – a population of non-identical oscillators responding slowly to GABA in a phase-dependent manner This model successfully reproduce the two key properties of the SCN self-organized synchronization and entrainment to external, cyclic stimuli. Taken all together, we propose that phase-dependent, slow response of clock cells to GABAergic inputs are the basis of the synchronization and entrainment of SCN neurons.

To see if GABA released endogenously from SCN neurons *in situ* plays an important role in synchronizing clock cells, we examined the effect of $GABA_A$ receptor antagonist bicuculline on the spontaneous firing activity of the ensemble of SCN neurons in rat hypothalamic slices. Figure 1 shows firing rates of the SCN neurons vs. projected zeitgeber time (ZT) of the animal colony both in control and bicuculline-treated conditions. In each of the control slices that were examined, a clear circadian rhythm was present in the ensemble activity of SCN neurons: the firing rate was significantly higher during the light (4.1 $\pm$ 0.2 Hz for ZT 4-8 hr; n–5) than dark phase (1.6 $\pm$ 0.2 Hz for ZT 16-20 hr; n–5) On the other hand, in slices bathed in 30-μM bicuculline containing medium for two days, such a rhythm was absent. the firing rate during the light phase (3.3 $\pm$ 0.3 Hz for ZT 4-8 hr; n=6) was not significantly different from that during the dark phase (3.1 $\pm$ 0.1 Hz for ZT 16-20 hr; n–5) These results indicate that GABA receptor-mediated synaptic transmission is indeed critical for the clock cell synchronization.

In order to understand the nature of the GABA mediated intercellular communication, extracellular single-unit recordings were made simultaneously from various pairs of neighboring neurons (157 pairs in 14 slice preparations) Initially, the cross-correlograms of their spiking activities were examined with the maximum range of $\pm$ 1 sec and bin size of 20-200 ms. Only 6 out of 157 pairs exhibited a significant peak (or trough) in the cross-correlogram, while the other pairs did not show any correlation (data not shown) In other words, fast spike timing correlation turned out to be quite rare among the neighboring SCN neurons.

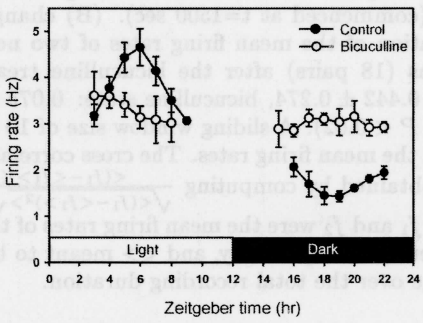

FIG 1 Effect of bicuculline on the circadian rhythm of spontaneous firing activity of SCN neurons. The 2-hr running averages of the firing rates (with 1-hr lag) of randomly sampled SCN neurons in control (closed circles) and bicuculline-treated slices (open circles) are plotted against projected ZT All recordings were carried out on the third day in vitro. To avoid tissue run-down, each hypothalamic slice was recorded for only 7-9 hr duiring either the light or dark phase. The bicuculline treatment of the slice was commenced right after the slice preparation and continued until the end of experiment.

Subsequently, time series of mean firing rates (see Fig.2A) were computed for all pairs of clock cells and their cross-correlations were examined. Of total 47 pairs that were recorded continuously over 30 minutes, 26 showed a significant cross correlation coefficient value (mean $\pm$ SEM. 0.549 $\pm$ 0.225, window size: 10 sec) when the window size for averaging was 10 sec or longer In other words, the relevant time scale of the SCN cell-

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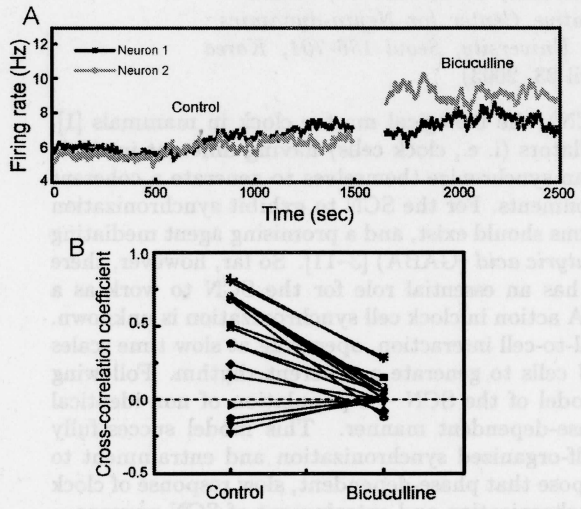

FIG 2 Effect of bicuculline on cell-to-cell communication in the SCN (A) typical time series of mean firing rates of two neighboring SCN cells before and during the bicuculline treatment (commenced at t=1500 sec) (B) changes in the cross correlation of the mean firing rates of two neighboring SCN neurons (18 pairs) after the bicuculline treatment (control state: 0.442 ± 0.274, bicuculline state: 0.075 ± 0.086, paired t-test, $P < 0.02$) A sliding window size of 10 sec was used to obtain the mean firing rates. The cross correlation coefficients were obtained by computing $\frac{<(f_1-<f_1>)(f_2-<f_2>)>}{\sqrt{<(f_1-<f_1>)^2>}\sqrt{<(f_2-<f_2>)^2>}}$, where f_1 and f_2 were the mean firing rates of the first and second neurons, respectively, and $<>$ meant to be the temporal average over the total recording duration.

Although we just proved that GABA-mediated synaptic transmission is essential for the synchronization of the clock cells and the relevant time scale of the GABA mediated cellular interaction is quite slow, it is still unclear how exactly GABA shapes the coherent output rhythm of SCN? At this point, we formulated a hypothesis that the coherent firing activity of the SCN arises from slow, phase-dependent, phase-shifting responses of the individual clock cells to GABAergic stimulus. That is, the circadian phase of the individual oscillator can be either advanced or delayed based on the circadian phase at which a GABA stimulation is given, and the stimulation is provided by the endogenous GABA released from neighboring cells.

Based on this hypothesis, we constructed a simple but novel model for the SCN (see Methods) a network of 10,000 self-sustained oscillators (with different intrinsic periods ranging $20 \sim 28$ hr), being coupled by the 'GABA phase response curve(PRC)' of Fig.3. According to this PRC, the circadian phase ϕ_i of each oscillator can be advanced (for ϕ_i $0 \sim \pi/2$ and $3\pi/2 \sim 2\pi$) or delayed

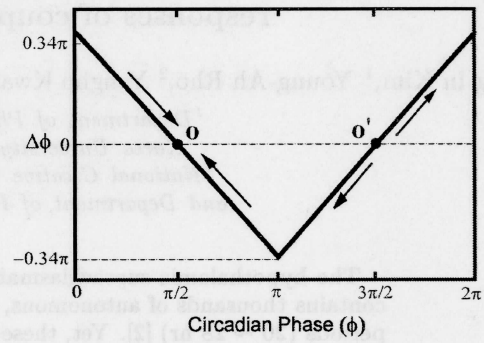

FIG 3: Model GABA phase response curve plotting the phase shift $\Delta\phi$ induced by a 'GABA stimulation' as a function of the circadian phase ϕ. A positive (negative) value of $\Delta\phi$ means an advance (delay)

(for ϕ $\pi/2 \sim 3\pi/2$) upon receiving a GABA stimulus from neighboring cells. The shape of this PRC was modeled after the recent experimental work [11] by Liu and Reppert who examined phase shifting property of cultured clock cells to exogenous GABA, applied daily for 1 or 6 hr In our model, the sum of the firing rates of the neighboring cells (A_i) assumed the role of endogenously released GABA. that is, the more frequently the SCN neurons fired, the greater the amount of GABA was released. Then, we further assumed that only when the sum A_i had stayed larger than a critical value (e. g., $A_c = 10,000$) for a sufficient period of time (e. g., $T_c = 1$ hr), the GABA action would take a part for the ith cell through the PRC of Fig. 3. The total sum A_{tot} of the firing rates of the individual oscillators was monitored as an output signal of the entire network.

Figure 4 well illustrates a typical example undergoing self-organized synchronization and subsequent entrainment to external, cyclic stimuli. The simulation initially began with an uniformly distributed phases (gray in Fig.4A) and a normal distribution of intrinsic periods (gray in Fig.4B) As the oscillators gradually synchronized to each other through the PRC-mediated cell-to-cell interaction, the network produced a coherent rhythm in A_{tot} with a period of 24.8 hr after a transient period of about 70 hr (see Fig.4C) For the final synchronized state, the distribution of the phases (black in Fig.4A) and that of the periods (black in Fig.4B) both showed a significant degree of coherence. The behavior of the coupled network stayed essentially the same with different values of A_c and T_c as long as they were within the range Ac $8,500 \sim 10,100$ and $T_c = 0.4 \sim 3.0$ hr Thus, the coupling interactions based on the V-shaped PRC can synchronize individual oscillators so as to produce a coherent output rhythm.

How can the coupling interaction mediated by a PRC like the one shown in Fig.3 synchronize the non-identical

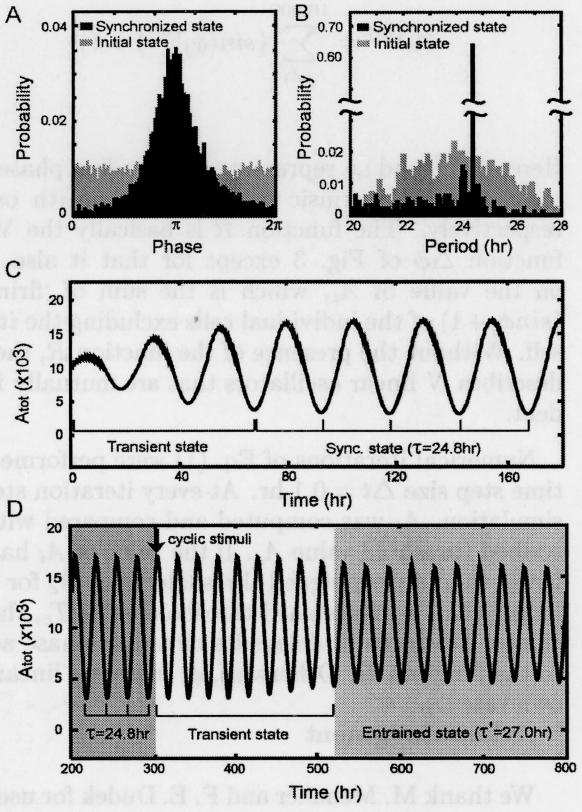

FIG 4: Synchronization and entrainment of the model SCN network. The distributions of phases (A) and periods (B) of the individual oscillators before and after the synchronization. (C) shows the emergence of a self-synchronized rhythm in the total sum of the firing rates (A_{tot}) (D) shows the entrainment of the self-synchronized network by external, cyclic stimuli (commenced at the position marked by an arrow)

oscillators? Our heuristic explanation involves two different physical issues: (1) *phase-locking* and (2) *frequency-lockıng.* The phenomenon of phase-locking is rather easy to understand if one recognize that the V-shaped PRC has two fixed points – a stable one O and an unstable one O' The point O (O') is stable (unstable) in the sense that all circadian states converges (diverges) to the point as indicated by the arrows, upon receiving a sequence of stimulations. In fact, PRCs having one stable fixed point always bring the phase of each oscillator of the network to a single value, thus resulting a stable phase-locked rhythm. In the mean time, the differences in the intrinsic periods of the individual oscillators disappear as the system undergoes frequency-locking, an universal phenomenon in nonlinear systems of coupled oscillators [12, 13] The nonlinearity of our model system originates from the nonlinear shape of the PRC function R and the existence of the threshold values, A_c and T_c

The same model also exhibited another important characteristic of the SCN – *entrainment.* The self organized output rhythm (period=τ) of the network could be entrained to the rhythm (period=τ') of the externally applied stimulus. For the case shown in Fig. 4D, for example, at every hour of a total 6-hour duration the phase of the each oscillator ϕ_i was forced to have a shift based on the function $R(\phi_i)$ regardless of the value of A_i Then, this protocol was repeated at every τ' 27.0 hr As a result, the period of the initial rhythm (τ 24.8 hr) gradually altered to match that of the stimulus (τ' 27.0 hr) The phenomenon of entrainment was consistently observed as long as $23 < \tau' < 27$ for the same stimulation protocol. The entrainment also occurred even if the external stimulus was delivered before the network had self-organized to produce a coherent rhythm.

We also investigated the minimum coupling range of GABA-mediated intercellular communication necessary for the synchronization of clock cells. The connectivity of our model network was systematically varied from 2 (i. e., nearest neighbor coupling) to 10,000 (i. e., all-to-all coupling) When the number of the connected oscillators were less than 20, no clear coherent rhythm emerged at all. But, a stable rhythm always existed when the number of connected oscillators were greater than 30. In other words, a certain level of intercellular connectivity was required for the oscillators to rhyme in a coherent manner This result may explain the lack of a coherent rhythm in the cultures of SCN cells [2], in low-density cultures the degree of GABA-mediated coupling can be quite low, although some functional GABAergic synapses are present [14]

The experimental evidence presented in this work supports that a GABA-mediated, slow cell-to-cell coupling interaction is essential for SCN clock cells to synchronize and generate a coherent output rhythm. Following this observation, we have introduced a new concept that the cell-to-cell coupling interaction of SCN is mediated by a phase response curve of GABA. Two essential properties of the SCN *in situ* self-organized synchronization and entrainment – are well captured in the model employing this idea.

Methods

Brain slice preparation

Hypothalamic slices (500-μm thick one for conventional extra cellular recording or 150-μm thick one for paired extra cellular recording) were cut from the brains excised from male Sprague-Dawley rats (150-200g, n=35), according to the method described in our previous report [15] Before the slice preparation, the animals had been housed in a temperature-controlled room (22 24°C) with 12/12 hr light-dark schedule for at least 10 days [ZT 0:00 hr (ZT 12:00) was defined as the time of lights-on (lights-off) in the animal colony] For each rat, one slice containing the core of SCN was selected, and transferred to a beaker filled with gentamicin (50 mg/l)-supplemented artificial cerebrospinal fluid (ACSF, composition in mM. 124.0 NaCl,1.3 $MgSO_4$ $7H_2O$, 3.0

KCl, 1.25 NaH_2PO_4, 26.0 $NaHCO_3$, 2.4 $CaCl_2$,10.0 glucose) For the bicuculline experiments, 30 μM (in ACSF) bicuculline methiodide was used. The medium was kept warm at 35°C and aerated continuously with a mixed gas (95% O_2, 5% CO_2)

Extracellular single-unit recordings

After two days of incubation in the beaker, the slice was transferred to a modified Haas-type gas interface recording chamber [16] that was perfused continuously with fresh medium at the rate of 0.5 ml/min With the use of glass electrodes [1-μm tip diameter; 4-5 $M\Omega$, filled with 3-M NaCl (pH 7.4)], conventional extracellular single-unit recordings were obtained from individual SCN neurons during projected light or dark phase. Typically 10 units were sampled for every hour (1-min recording time for each) The firing rates of the sampled units were grouped into a 2-hr running average with 1-hr lag to follow the circadian time-dependent fluctuation in the ensemble activity of SCN neurons. For the paired extracellular single-unit recordings, the cell-attached recording technique was used. A separate glass electrode (~2-μm tip diameter; 3-5 $M\Omega$, filled with ACSF) was attached to each cell of the targeted pair with weak mouth suction. Target pairs that were within 50 μm from each other were chosed randomly The simultaneous dual recording was made for 20-40 min.

Model network of the SCN

The dynamics of our model network is described by the following set of phase equations:

$$\phi_i^{\,m+1} \quad \phi_i^{\,m} + \omega_i \triangle t + R(\phi_i^{(m)}, A_i^{\,m}), \qquad (1)$$

$$A_i^{\,m} \quad \sum_{j \neq i}^{10,000} \{sin(\phi_j^{\,m}) + 1\} \qquad (2)$$

Here, $\phi_i^{\,m}$ and ω_i represent the circadian phase at time $m\triangle t$ and the intrinsic frequency of the ith oscillator, respectively The function R is basically the V-shaped function $\triangle\phi$ of Fig. 3 except for that it also depends on the value of A_i, which is the sum of 'firing rates' $(sin\phi_j + 1)$ of the individual cells excluding the ith cell itself. Without the presence of the function R, the Eq (1) describes N linear oscillators that are mutually independent.

Numerical iterations of Eq (1) were performed with a time step size $\triangle t$ 0.1 hr At every iteration step of the simulation, A_i was computed and compared with a prescribed threshold value A_c If the value of A_i had stayed larger than a preassigned threshold value A_c for a period longer than a preassigned threshold value T_c, the ith oscillator advances or delays its circadian phase according to the function R. Otherwise, ϕ_i increases linearly at its own rate ω_i

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