

Synchronization of circadian firing rhythms in cultured rat suprachiasmatic neurons

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Abstract

The circadian clock in mammals is located in the suprachiasmatic nucleus (SCN) which consists of multiple oscillating neurons. Integration of the cellular oscillations is essential for the generation of a single circadian period in the SCN. By using a multielectrode dish (MED), we measured circadian firing rhythms in individual SCN neurons for more than 2 weeks continuously, and examined the involvement of synaptic communication in the synchronization of circadian rhythms. Cross-correlation analysis of spontaneous action potentials revealed that a neuron pair was functionally connected by synapses when their circadian rhythms were synchronized. No correlation was found between the paired neurons whose circadian rhythms were not synchronized. Calcium (Ca^{2+})-dependent synaptic transmission in the cellular communication was indicated by dose-dependent lengthening of an intercellular spike interval and loss of spike correlation with a Ca^{2+} channel blocker. Approximately 60% of the SCN neurons in culture were immunoreactive to antibodies against γ -aminobutyric acid (GABA) or glutamic acid decarboxylase (GAD). Spontaneous firing of all the neurons tested was either increased or decreased by bicuculline, the GABA_A receptor antagonist. These findings indicate that synaptic communication plays a critical role in the synchronization of circadian rhythms in individual SCN neurons and the GABAergic transmission is involved in the synchronization mechanism.

Introduction

The circadian clock in the suprachiasmatic nucleus (SCN) entrains to a 24-h light–dark cycle and regulates the temporal organization of physiological functions (Moore & Eichler, 1972; Stephan & Zucker, 1972). In the absence of external time cues, the SCN generates an endogenous single circadian period *in vivo* (Inouye & Kawamura, 1979). The SCN in rats contains about 8000 neurons (van den Pol, 1980). Recently, single SCN neurons were demonstrated to show circadian firing rhythms with different periods, indicating that many SCN neurons have their own circadian oscillator (Welsh *et al.*, 1995; Liu *et al.*, 1997; Herzog *et al.*, 1998; Honma *et al.*, 1998a). Therefore, the multiple oscillators must be integrated within the SCN to generate a single circadian period.

With respect to the synchronization mechanism of circadian rhythms among individual SCN neurons, calcium spikes, paracrine–hormonal interaction, electrical coupling via gap junctions and ionic interactions have been suggested (Bouskila & Dudek, 1993; van den Pol & Dudek, 1993; Jiang *et al.*, 1997). On the other hand, contribution of synaptic communication to the rhythm synchrony is questioned because day–night variation of SCN metabolic activity is detected prenatally before the initiation of synaptogenesis (Reppert & Schwartz, 1984; Shibata & Moore, 1988). However, the finding does not necessarily deny the role of synaptic transmission in the rhythm synchrony, because individual oscillation in the fetal SCN neurons may be separately entrained by time cues, such as maternal rhythms. The number of synapses in the SCN markedly increases from

postnatal day 4 to day 10 and neural networks continue to develop with the extension of dendritic processes (Moore & Bernstein, 1989). In rat brain it is well established that synaptogenesis occurs in a few days and neural networks which can induce synchronized bursting develop progressively (Kamioka *et al.*, 1996; van den Pol *et al.*, 1998). Inhibitory synaptic currents were frequently detected in cultured SCN neurons by whole-cell patch recordings (Welsh *et al.*, 1995), although interaction of spontaneous spike discharge was not confirmed among individually oscillating neurons in this study. Thus, the function of the synaptic communication among SCN neurons is still unknown.

By using a multielectrode dish (MED), we measured circadian firing rhythms in 4–8 neurons simultaneously and demonstrated that individual SCN neurons exhibited robust circadian firing rhythms (Honma *et al.*, 1998a). In the present study, we found pairs of SCN neurons that showed synchronized circadian firing rhythms. Temporal correlation of spontaneous discharges among SCN neurons on a MED was studied by means of cross-correlation analysis (Toyama *et al.*, 1981; Huntsman *et al.*, 1999). The method is noninvasive and enables us to analyse the synaptic mechanism of rhythm synchronization among individual SCN neurons. In addition, properties of the synaptic transmission were examined by using a Ca^{2+} channel blocker and a GABA_A receptor antagonist.

Materials and methods

SCN cell culture

Four-day-old Wistar rats from an inbred colony were anaesthetized by hypothermia and decapitated in the daytime. SCN cells were dissociated as described previously in detail (Honma *et al.*, 1998a,

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1998b) and cultured by plating $\sim 1.0\text{--}1.5 \times 10^5$ vial cells in the central area of a MED. The SCN cells were incubated with 1 mL of culture medium at 37 °C, 5% CO₂ and 95% air with saturating humidity throughout the experiment.

Spike recording

Extracellular action potentials of single neurons were recorded simultaneously by a multichannel recording system (Panasonic, Kyoto, Japan). The MED contains 64 planar embedded microelectrodes ($50 \times 50 \mu\text{m}$ in size) which were arranged in an area of 1 mm^2 . Recording of spontaneous firing was started at 4–10 days after the start of the culture and continued for 2–15 weeks. Seventy-two dishes were used for the experiments. Neurons ($n=4\text{--}8$) on the same dish were selected for measurement using the criteria of S/N ratio (>5.0). Spike discharges were fed through window discriminators (Nihon Koden, Tokyo, Japan) to a microcomputer and the number of spikes was counted every 10 s.

Analysis of circadian rhythm and cross-correlation

Circadian rhythm was analysed using a sequential data array of the mean firing rate over 15 min. The circadian period was calculated over 20–28 h by the chi-square periodogram (Sokolove & Bushell, 1978) using the data of at least five consecutive days. Neurons which had a single and statistically significant circadian period ($P < 0.01$) were designated as those with circadian oscillation. Cross-correlation was calculated by a micro1401 interface (CED, Cambridge, UK) for spike discharges collected with a multichannel data recorder (TEAC, Tokyo, Japan) at different times of the day. A spike discharge from one of the paired neurons was used as a reference, and the timing of spikes from the other neuron was examined over the range within ± 1 s. This process was repeated until the number of reference spikes exceeded 500 (maximum 2000). A histogram consisting of cumulated spike numbers plotted against time was constructed (cross-correlogram). To detect the excitatory synaptic communication, the resolution limit was set at $100 \mu\text{s}$ and the inhibitory communication at 1 or 10 ms. An intercellular spike interval (ISI) was obtained from the cross-correlogram of the paired neurons with excitatory communication. The possibility of reoccurring spikes originated from different processes of the same neuron was excluded by confirming that some of the spikes from one electrode occurred independently of those from the other.

Pharmacological treatment

Cadmium chloride (CdCl₂; Sigma, St Louis, MO, USA) and bicuculline (RBI, Natick, MA, USA) were dissolved in distilled water and mixed with the culture medium. The volume of the test solution added to the medium was $\leq 5 \mu\text{L}$. Increasing doses of the drugs were added every 4–5 min. We judged that the efficiency of the synaptic communication was affected by a pharmacological treatment when a neuron pair showed >0.2 ms lengthening as well as $>10\%$ increase in the ISI.

Immunohistochemistry

Immunohistochemistry for glutamic acid decarboxylase (GAD) was carried out as follows. The SCN cells cultured on the MED were fixed with 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4) for 45 min. After incubating with mouse monoclonal anti-GAD antibody ($1 \mu\text{g}/\text{mL}$; Boehringer Mannheim, Mannheim, Germany) at 4 °C for 40 h, the cells were processed by the standard avidin–biotin–

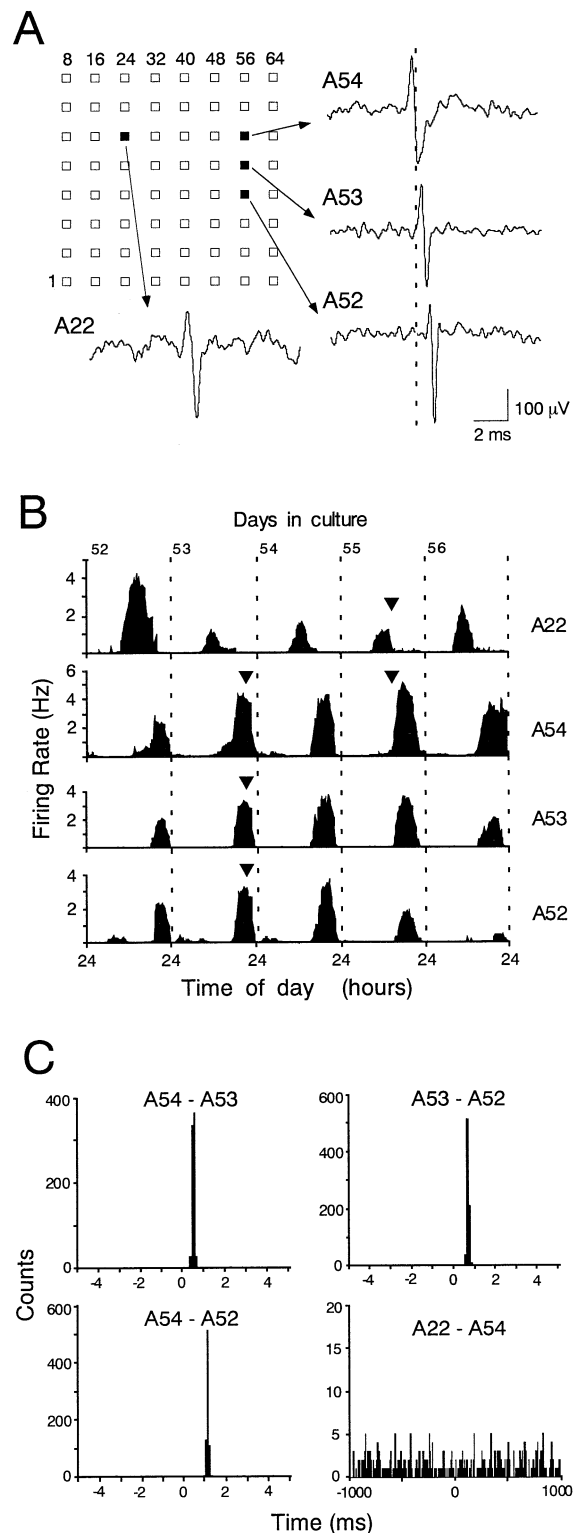


FIG. 1. Spontaneous discharges and circadian variations in firing rate in four neurons on a MED. (A) Diagram of the MED and representative spike waveforms recorded from neurons A54, A53, A52 and A22. The interpolar distances from A54 to A53, to A52, and to A22 were 150, 300, and 600 μm , respectively. (B) Synchronization and desynchronization of circadian firing rhythms in the four neurons. The circadian rhythm is expressed as the mean firing rate (Hz) in 15 min. The triangles in the graph indicate the time when the cross-correlations were analysed. (C) Cross-correlograms. Correlations that indicate excitatory synaptic communications were present between the neurons (A54, A53, A52) whose circadian rhythms were synchronized. The abscissa is the timing of spikes with bins of $100 \mu\text{s}$ (except for A22–A54) and of 10 ms (A22–A54), and the ordinate is the cumulative number of spikes.

peroxidase method (Vectastain Elite ABC Kit; Vector, Burlingame, CA, USA) using diaminobenzidine tetrahydrochloride (Dojin, Tokyo, Japan) as a chromogen (Honma *et al.*, 1998b). For GABA immunostaining, the SCN cells cultured on the MED were fixed with 1.75% glutaraldehyde and 2% paraformaldehyde in 0.1 M cacodylate buffer (pH 7.4) for 10 min. After incubating with rabbit polyclonal anti-GABA antibody (1 : 200; Chemicon, Temecula, CA, USA) at 4 °C for 48 h, the cells were processed by the avidin–biotin–peroxidase method as described above.

Results

SCN neurons show synchronized circadian firing rhythms

Spontaneous action potentials in individual SCN neurons became obvious as early as the fourth day in the dissociated cell culture on the

MED. Figure 1 shows spontaneous spike discharges and circadian variations in firing rate measured simultaneously in four neurons on the same MED. Three neurons (A52, A53, A54) showed synchronized circadian rhythms with a period of 23.7 h (Fig. 1B). The A22 neuron exhibited the circadian rhythm with a different period (23.0 h), which phase-led the other three by 7 h at the beginning of the recording. Cross-correlograms (Fig. 1C) indicate a unidirectional excitatory communication between A54–A53, A53–A52, and A54–A52. The former neurons (A54, A53, A54) in the three pairs are used as a reference. ISI, an interval between the reference time (0 ms) and the peak in the correlogram, was 0.5 ms (A54–A53), 0.7 ms (A53–A52) and 1.2 ms (A54–A52), respectively. In contrast, there was no correlation in the timing of spikes between A22 and A54 (and between A22 and the others; data not shown), indicating a lack of synaptic communication.

TABLE 1. Relationship between synchronization of circadian firing rhythms and synaptic communication

	Rhythm(+/+)		Rhythm(+/-)	Rhythm(-/-)	Total
	Synchro(+)	Synchro(-)			
Numbers of neuron pairs					
Correlation (+)	45	0	43	50	138
Correlation (-)	0	81	123	140	344
Total	45	81	166	190	482

Numbers of neuron pairs are shown. In total, 264 neurons were examined and, of these, 123 showed circadian firing rhythm throughout the experiment. When the circadian periods of firing rhythms in a paired neurons are identical, the circadian rhythms are regarded as synchronized [synchro(+)]. Rhythm(+/+), both of the neurons showed circadian firing rhythms; rhythm(+/-), one neuron showed a circadian rhythm but the other did not; rhythm(-/-), none of the paired neurons showed a circadian rhythm.

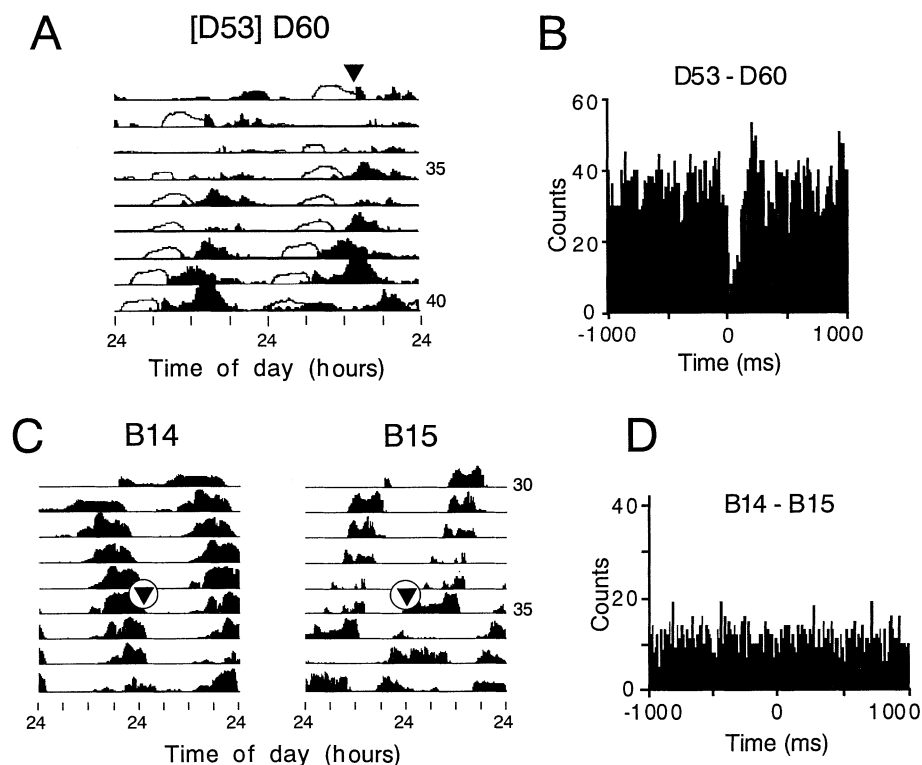


FIG. 2. Circadian firing rhythms of paired neurons and their cross-correlograms. (A) Double-plotted circadian firing rhythms of neurons with inhibitory synaptic communication. These rhythms were synchronized with a phase-lag of about 6 h and the circadian period of both neurons was 23.6 h. The rhythm of D53 (outlines) is superimposed on that of D60. Triangles indicate the time when the cross-correlation was analysed. The numbers at the right side of the graph indicate the number of days in cell culture. The full scale of firing rate, 8 (Hz, D53) and 6 (D60). (B) A cross-correlogram between D53 and D60. Here, D53 exerted a synaptic inhibition on D60. Suppression of the action potentials of D60 was observed from 4 to 140 ms after the reference spikes of D53. The two neurons were located on adjacent electrodes. (C) Double-plotted circadian rhythms of two neurons on the same MED. These rhythms were not synchronized. The circadian period of B14 was 25.2 h and that of B15 was 22.8 h. Triangles in the graph indicate the time when the cross-correlation was analysed. The full scale of firing rate, 7 (B14) and 4 (B15). The two neurons were located on adjacent electrodes. (D) An absence of the cross-correlation in the neuron pair B14 and B15.

Table 1 summarizes the relationship between the synchronization of circadian rhythms and the synaptic communication in 482 neuron pairs. One hundred and twenty-six neuron pairs displayed circadian firing rhythms in both neurons and, of these, 45 showed synchronized circadian rhythms. In the neuron pairs with synchronized circadian rhythms, either excitatory or inhibitory communication was demonstrated without exception. Forty-two pairs had an excitatory communication, and the remaining three displayed an inhibitory communication. In the synchronized neuron pairs with inhibitory communication, neuronal firing was suppressed for 100–140 ms after the reference time (Fig. 2A and B). Interestingly, these neurons showed a large phase-angle difference in the circadian rhythms. In 81 of 126 pairs, circadian rhythms were not synchronized. We did not detect any synaptic communication among these neuron pairs (Fig. 2C and D).

Synaptic delay and neuronal conduction velocity

The ISI of the excitatory communication ranged from 0.1 to 5.0 ms in the neuron pairs with synchronized circadian rhythms. Ten neuron pairs of 42 showed an ISI of <0.3 ms. The relation between the ISI and intercellular distance was analysed in 32 pairs with the ISI of 0.3 ms or longer (Fig. 3). Because neuron pairs can be connected either mono- or polysynaptically, they were divided into two groups according to their ISI (i.e. shorter or longer than 2 ms; Toyama *et al.*, 1981). The ISI was strongly correlated with the distance between the paired neurons in both groups. Neuron pairs on the lower regression line had a synaptic delay of 0.3 ms and a conduction velocity of 0.38 m/s. Neuron pairs on the upper regression line had a synaptic delay of 2.2 ms and a conduction velocity of 0.33 m/s.

Properties of the synaptic communication

The nature of neural communication in the cultured SCN neurons was tested by applying Cd^{2+} , a nonselective Ca^{2+} channel blocker (Müller *et al.*, 1992). Of the 12 synchronized pairs examined, cross-correlation was lost in four pairs at doses of $1 \mu\text{M}$ Cd^{2+} . Dose-dependent lengthening of the ISI was detected in the remaining eight pairs (Fig. 4). In three of them, the correlation was lost with $50 \mu\text{M}$ Cd^{2+} . Spontaneous firing rate was changed by Cd^{2+} application in nine of ten neurons tested. Seven neurons showed an increase and two showed a decrease in firing rate with $50 \mu\text{M}$ Cd^{2+} .

The involvement of neural communication through GABA was investigated. About 60% of the neurons ($56.9 \pm 1.0\%$, mean \pm SEM, $n=5$) were immunoreactive to antibodies against either GABA or GAD (Fig. 5A). The GAD-immunoreactive axon varicosities were closely associated with dendrites and somata of the other neurons. Bicuculline, a GABA_A receptor antagonist, was added to the medium and the responses of the spontaneously firing neurons ($n=43$) on the MEDs ($n=7$) were examined. The spontaneous firing rate was increased in 36 neurons and decreased in seven at $10 \mu\text{M}$, and increased in 40 and decreased in three at $50 \mu\text{M}$ bicuculline.

The effect of 10–100 μM bicuculline on the spontaneous firing activity was further examined in another 11 neurons consisting of seven pairs with synchronized rhythms. The spontaneous firing was decreased dose-dependently in three and increased in eight neurons. By the application of bicuculline, ISI was either lengthened or abolished in two pairs but was unchanged in the remaining five pairs. In one of the former three neurons, the firing rate was decreased by bicuculline when the spontaneous firing rate was high (active phase), but was increased when the firing rate was low (inactive phase; Fig. 5B and C). Vehicle treatment (5 μL of distilled water) had no effect on the spontaneous firing rate nor on the ISI.

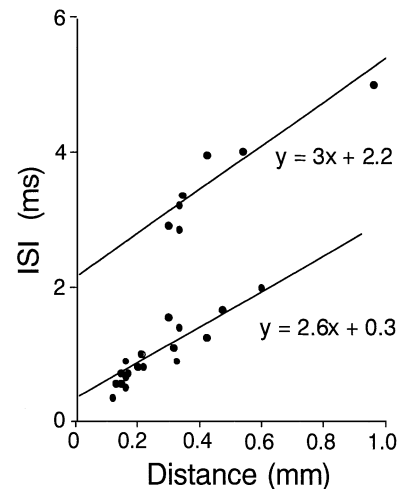


FIG. 3. Relationship of the ISI to the distance between the paired neurons with synchronized circadian rhythms. The oblique lines in the graph indicate the regression lines obtained from two groups of the neuron pairs. Correlation coefficient of the neuron pairs on the lower regression line and the upper regression line was 0.789 ($n=25$, $P<0.0001$) and 0.934 ($n=7$, $P<0.001$), respectively.

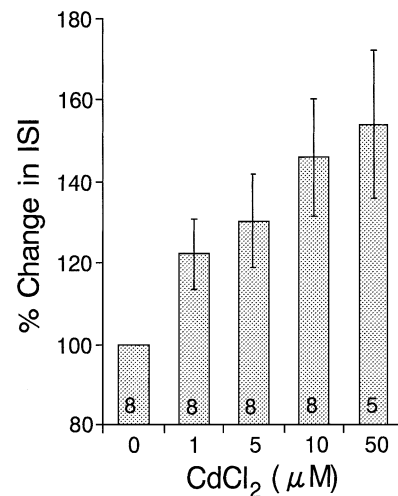


FIG. 4. Dose-dependent lengthening of the ISI by Cd^{2+} ($P<0.002$, one-way ANOVA of repeated measures). The ordinate indicates ISI expressed as percentage of the pretreatment value (mean \pm SEM). The number in the column indicates number of neuron pairs analysed.

Discussion

Our data demonstrate that the SCN neurons cultured on the MED were functionally connected by synapses when their circadian rhythms were synchronized. Cross-correlograms indicate a unidirectional communication between the neurons with synchronized circadian rhythms (Fig. 1C). Forty-five neuron pairs of 482 showed synchronized circadian rhythms and exhibited either excitatory or inhibitory communication without exception. We did not detect any synaptic communication among neuron pairs whose circadian rhythms were not synchronized, which is consistent with a previous report by Welsh *et al.* (1995). In their study, however, synchronized circadian rhythms were not observed in any neuron pair, in marked contrast to our findings where in 36% of neuron pairs the circadian rhythms were synchronized.

In the neuron pairs with excitatory communication, Cd^{2+} either lengthened the ISI or abolished the cross-correlation (Fig. 4). Cd^{2+} is

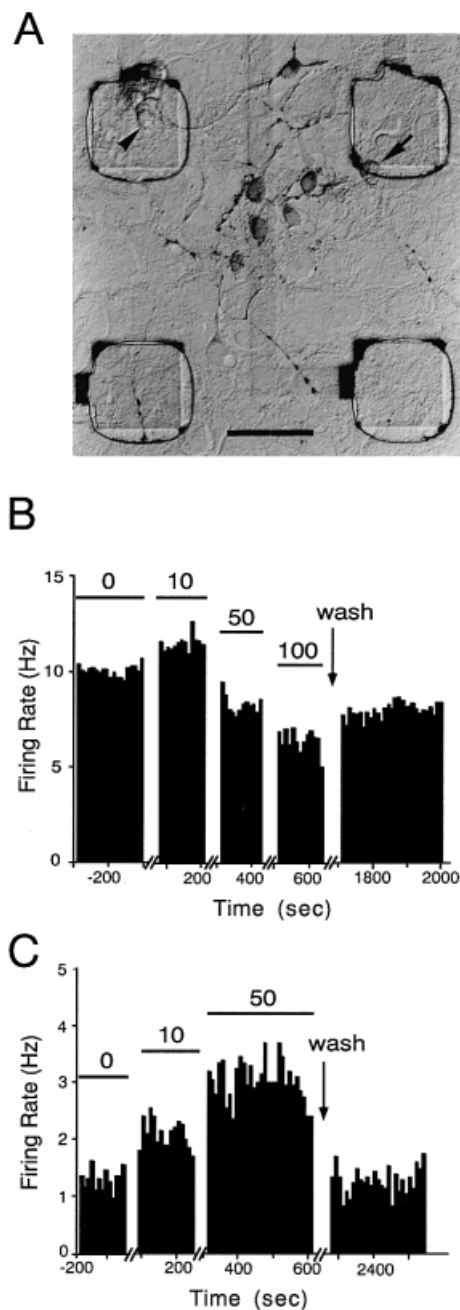


FIG. 5. GAD-immunoreactive neurons and responses of a neuron to bicuculline. (A) Photomicrograph of GAD immunohistochemistry. The SCN neurons on a MED were fixed on the 14th day of culture. Most neurons in the micrograph are GAD-positive. Four electrodes appear as squares located at regular intervals (interpolator distance 150 μ m). An arrow and arrowhead indicate GAD-positive and -negative neurons on the electrode, respectively. Scale bar, 50 μ m. (B and C) Effects of bicuculline on spontaneous firing activity of a single SCN neuron which showed circadian rhythm. Dose-dependent decrease in the firing rate at the active phase (B) or increase at the inactive phase (C) was observed in the same neuron measured 8 days apart. Concentrations of bicuculline (μ M) are indicated above the horizontal bars. Blanks between the firings in these records correspond to the time when the MED was offline. The abscissa indicates the time after the start of the bicuculline application.

known to block the voltage-activated Ca^{2+} channels and suppress the synaptic transmission (Müller *et al.*, 1992). The velocity of synaptic transmission was correlated with the Ca^{2+} entry in the presynaptic terminal, and the synaptic delay became longer when the Ca^{2+} entry

was decreased (Sabatini & Regehr, 1996). The present result indicates that the intercellular communication is synaptic. In the 42 neuron pairs with synchronized circadian rhythms, the ISI varied from 0.1 to 5 ms. Among them, 10 pairs with the ISI shorter than 0.3 ms were regarded as receiving common inputs (Toyama *et al.*, 1981). In the remaining 32 pairs, the ISI was strongly correlated with the distance between the paired neurons (Fig. 3). The conduction velocity calculated from the ISI and intercellular distance was close to the value previously measured in unmyelinated fibers (0.55 m/s; Lee *et al.*, 1986) in both groups with mono- and polysynaptic connections.

Most SCN neurons contain GABA (Moore & Speh, 1993) which is generally regarded as an inhibitory neurotransmitter (Kim & Dudek, 1992; Strecker *et al.*, 1997; Gribkoff *et al.*, 1999). In the present experiment, about 60% of neurons on the MED were either GABA- or GAD-positive, which was a similar rate to that reported by Welsh *et al.* (1995). Bicuculline increased the spontaneous firing rate in most of the neurons examined, indicating that the GABAergic transmission is primarily inhibitory. On the other hand, as long as the neuron pairs with synchronized rhythms were analysed, more than 90% of the synapses were found to be excitatory. About 80% of the excitatory communications had a short (<2 ms) ISI and the synaptic delay was calculated to be 0.3 ms. The predominance of excitatory communication may not be explained by excitation through disinhibition of the GABAergic inputs, because the estimated synaptic delay is in the range of monosynaptic communication at physiological temperature (Appenteng *et al.*, 1989; Borst *et al.*, 1995; Sabatini & Regehr, 1996). GABA has been shown to act as an excitatory transmitter (Alger & Nicoll, 1982; Nicoll *et al.*, 1990; Michelson & Wong, 1991; Kaila, 1994; Chen *et al.*, 1996; Wagner *et al.*, 1997). Especially, in the SCN, GABA increased the spontaneous firing rate during the subjective day while decreased it during the subjective night which was explained by shifts in the chloride equilibrium potential (Wagner *et al.*, 1997). Although the findings are not necessarily confirmed (Gribkoff *et al.*, 1999), bicuculline decreased spontaneous firing dose-dependently in some neurons in our culture, indicating that GABA can act as an excitatory neurotransmitter. Furthermore, one neuron decreased its firing rate in response to bicuculline at the active phase while increased it at the inactive phase (Fig. 5B and C). The result indicates that the responsiveness of the neuron to GABA is phase-dependent. Cross-correlation analysis was performed mostly during the active phase which corresponded to the subjective day when GABA was reported to excite SCN neurons (Wagner *et al.*, 1997). Therefore, the apparent discrepancy between the abundance of GABAergic neurons in the SCN and the predominance of excitatory communication in the neuron pairs with synchronized rhythms can be explained at least partly by excitatory transmission through GABA. Other mechanisms mediating excitatory intercellular communication among SCN neurons have been suggested (Bouskila & Dudek, 1993; van den Pol & Dudek, 1993). In the present experiment, ten neuron pairs with synchronized circadian rhythm had very short ISIs of <0.3 ms. Although they can be regarded as receiving common inputs, possible roles of electrical coupling are not excluded in the cellular communication because coupling through gap junctions has been identified in SCN neurons (Jiang *et al.*, 1997). In addition, glutamatergic synapses, which are generally known to mediate fast excitatory transmission, could be concerned with the mechanism, although glutamatergic neurons have not yet been confirmed in the SCN (Strecker *et al.*, 1997). Further experiments are necessary to clarify this issue.

In conclusion, synaptic communication plays a critical role in the synchronization of circadian rhythms in individual SCN neurons.

GABA, which is demonstrated to act as either an inhibitory or excitatory transmitter, is involved in the synchronization mechanism.

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Abbreviations

GAD, glutamic acid decarboxylase; ISI, intercellular spike interval; MED, multielectrode dish; SCN, suprachiasmatic nucleus.

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