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Monitoring the Clock Neuron's Tick: Circadian Rhythm Analysis Using a Multi-Electrode Array Dish

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17.1 Introduction

Almost all physiological and biochemical functions in the body oscillate with a period of approximately 24 h. These rhythms are not direct reactions to either environmental temperature fluctuations or the 24 h light–dark cycle, but are driven by an endogenous oscillator called the circadian clock, or simply "biological clock." Bodily functions even oscillate under unchanging environmental conditions such as constant darkness, though with a period close to, but significantly different from, 24 h. In fact the etymology of the term "circa (about)-dian (a day) rhythm" is derived from these many observations. For most organisms environmental light resets the intrinsic period exactly to 24 h. In mammals the master circadian clock resides in the suprachiasmatic nucleus of the hypothalamus (SCN; Moore and Eichler, 1972; Stephan and Zucker, 1972; Figure 17.1a). SCN neurons generate circadian periodicity and receive photic signals to adjust the intrinsic period to the 24 h day–night cycle. Thus they coordinate and orchestrate peripheral clocks in most organs and tissues throughout the body, expressing circadian rhythms in physiology and behavior as manifested by the sleep and wake cycle.

In traditional slice physiology, cellular or network oscillations occur on the order of milliseconds; however, measuring circadian periodicity of SCN neurons requires analysis on the order of days or even weeks. The multi-electrode array dish (MED) probe enabled us to monitor the activity of single SCN neurons long enough for circadian rhythm analysis. In this chapter we review the functions of "clock neurons", the SCN neurons with an intrinsic circadian oscillator, as unveiled by the use of the MED probe. We also include the methods we developed for the long-term culture of neurons and the analysis of time-series data.

17.2 Master Circadian Clock in the SCN

The SCN comprises bilaterally paired nuclei, each containing only ~8000 neurons within a volume of ~0.15 mm^3 (van den Pol, 1991). It receives direct photic

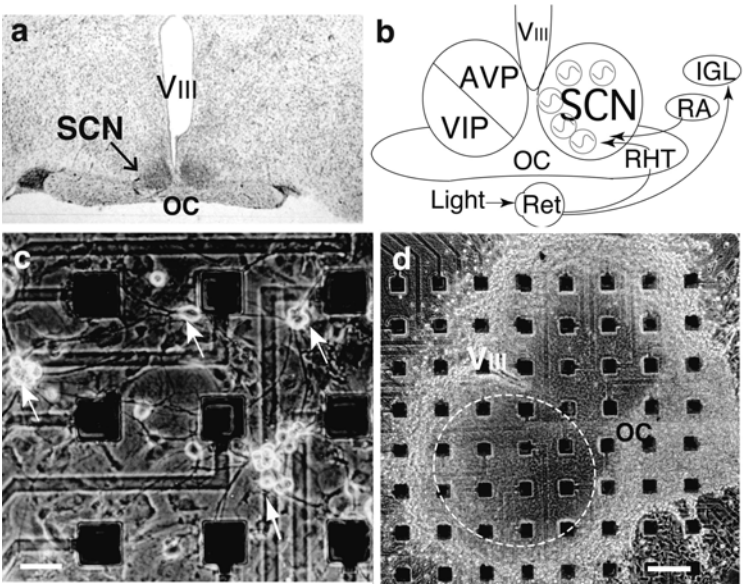

FIGURE 17.1. Photomicrograph and scheme of the SCN. (a) Coronal section of the rat hypothalamus stained with cresyl violet at the level of the SCN (arrow). OC: optic chiasm, V_{III}: third ventricle. (b) Schematic drawing of the rat SCN showing peptidergic neuron localization in the SCN and major afferent projections (arrows). : clock neurons, AVP: arginine vasopressinergic neuron (predominated region), VIP: vasoactive intestinal polypeptidergic neurons (predominated region), IGL: intergeniculate leaflet of the thalamus, Ret: retina, RHT: retinohypothalamic tract, RA: raphe nucleus in the midbrain. (c) Dispersed cell culture of the rat SCN on the MED probe at day 14 in culture. In this dish, SCN cells were seeded at the low concentration of 1000 vial cells/mm^3. White arrows: SCN neurons. Black squares: electrodes (50 μm × 50 μm). Scale: 50 μm. (d) An organotypic slice culture of the rat SCN at day 11 in culture. Broken oval lines: border of the SCN. Scale: 150 μm. (Honma et al., unpublished data).

inputs from the retina through the retinohypothalamic tract (Moore, 1973). The major photoreceptor responsible for the resetting of the circadian clock was recently found to be melanopsin, an opsin-based photopigment in retinal ganglion cells (Berson et al., 2002). The SCN is histologically and functionally divided into two subdivisions, the dorsomedial (shell) and ventrolateral (core). Major external afferents, those from the retina, intergeniculate leaflet, and raphe nucleus, project into the ventrolateral SCN where vasoactive intestinal polypeptide (VIP)-containing neurons predominate. On the other hand, in the dorsomedial SCN where arginine vasopressin (AVP)-containing neurons predominate, afferents from outside the SCN are limited, but there is dense innervation from the ventrolateral SCN. Major efferents from the SCN project to the subparaventricular zone (SPZ), though dorsomedial and ventrolateral neurons project differentially (van den Pol, 1991; Moore, 1996). Interestingly, efferents project mostly to the immediate periphery

of the SCN, whereupon circadian signals seem to be integrated and transmitted through secondary neurons.

In the SCN there are robust circadian rhythms in the synthesis and release of neuropeptides in vitro (Shinohara et al., 1995) as well as in vivo (Schwartz and Reppert, 1985). The multi-unit activity of the SCN continuously monitored from freely moving animals exhibits a robust circadian rhythm with a peak during the subjective day irrespective of the subjects' diurnality or nocturnality. If the SCN is surgically isolated as a hypothalamic island from the rest of the brain, the rhythm in the SCN continues, but the firing rhythm outside the hypothalamic island and also behavioral rhythms are both abolished. This indicates that the circadian pacemaker resides within the SCN itself (Inouye and Kawamura, 1979; Sato and Kawamura, 1984).

Until recently, it had been difficult to demonstrate whether the circadian rhythm was driven by single pacemaker neurons or emerged from neural networks within the SCN, because single-unit activity could not be monitored for sufficiently long durations for circadian rhythm analysis. This problem was finally solved by the use of the multi-electrode array system. Welsh et al. (1995) reported that activity rhythms of individual dispersed SCN neurons free-ran with a period close to 24 h. He also showed that tetrodotoxin (TTX), a sodium channel blocker, suppressed spontaneous firing of single SCN neurons but did not affect intracellular circadian oscillation, for the firing rhythm re-emerged after the washout of TTX at the phase expected by the rhythm before the TTX treatment. The data clearly demonstrated that the circadian rhythm in the SCN emerges not from a neuronal network but from individual pacemaker neurons. With the advent of the MED probe, the dream of assessing the mammalian biological clock via week-long or month-long analysis of neuronal SCN rhythms had finally become a reality.

17.3 Long-Term Monitoring of Clock Neurons' Tick: Two Types of SCN Cultures

To examine the characteristics of clock neurons, we cultured rat and mouse SCNs on the MED probes using two different culture systems: the dispersed cell culture for examining the functions of single SCN neurons and cell-to-cell interactions, and the organotypic slice culture for assessing regional specificity within the SCN and the role of SCN cell assemblages. The following sections describe these two systems.

17.3.1 Initial Preparation of the MED Probes

For initial preparation, careful cleaning and sterilization are necessary. Thereafter, the probe can be kept under clean conditions in a sterilized disposable Petri dish (100 mm diameter). On the day before the culture, the probe's dish surface is treated with brief repeated exposures to blue flame to increase its hydrophilicity.

For the dispersed cell culture, the dish surface is then precoated with 0.02% poly-L-ornithine overnight in 4°C to facilitate cell attachment (Honma et al., 1998a; Shirakawa et al., 2000). After rinsing with sterile distilled water (SDW), the probe is kept covered in culture medium supplemented with 2∼5% serum for at least 2 h in a CO_2 incubator.

On the other hand, for the organotypic slice culture, after blue-flaming, the probe surface is coated with a collagen gel sheet (Type IC collagen, Nitta Gelatin, Japan), which is then firmed by incubating at 37°C for 2∼3 h.

17.3.2 Dispersed SCN Cell Culture on the MED Probe

For the dispersed cell culture (Figure 17.1b), rat and mouse pups 3 to 5 days old are decapitated under hypothermic anaesthesia. Coronal hypothalamic slices of 600 μm and 350 μm thick for rats and mice, respectively, are obtained using a tissue chopper (McIlwain), and the SCN is dissected in ice-cold Preparation Buffer (Table 17.1a) under a dissecting microscope (Honma et al., 1998a; Shirakawa et al., 2000). Depending on experimental design and litter size, SCN blocks of roughly 3 to 30 pups are pooled together. The SCNs are incubated in 0.03% trypsin in Preparation Buffer at 37°C for 15 min. After rinsing the SCNs with Preparation Buffer containing 0.022% trypsin inhibitor and 0.01% DNase, SCN cells are dissociated by flushing with a fire-polished Pasteur pipette. The resulting cell suspension is filtrated with a #200 stainless mesh and centrifuged at 1500 rpm for 5 min. The dispersed SCN cells are resuspended with DMEM supplemented with 5% fetal bovine serum at a concentration of $\sim 10^6$cells/ml and seeded in the central area of the probe at the relatively high density of 5000∼7500 vial cells/mm^2. Figure 17.1b shows dispersed SCN cells seeded on a MED probe at a low density in order to show the neurons clearly.

Each dish is cultured with 1 ml of DMEM supplemented with 20× Supplement Solution (Table 17.1b) at 5% and fetal bovine serum at ∼2%. Spontaneous

TABLE 17.1. Composition of preparation buffer and supplements for culture medium.

a. Preparation Buffer (pH 7.3/0°C)	
NaCl	8.6 (g/liter ultrapure water)
KCl	0.3
HEPES	4.7
Na HCO_3	3.0
Kanamycin	0.02

b. 20× Supplement Solution (Mix with culture medium at 5% immediately before use)	
Apotransferrin	2 mg/ml
Insulin (water soluble)	10 μg/ml
Putrescine hydrochloride	2 mM
Progesterone (water soluble)	20 nM
Sodium selenite	0.6 μM

discharges from dispersed SCN cell cultures are monitored from 4 days up to 5 months. By supplementing the culture medium with a low concentration of serum (0.5∼2.0%), cell mobility can be minimized over the duration of the culture. To reduce serum-induced glial overgrowth, the culture is additionally treated with antimitotics (1 μM each of cytosine arabinoside, uridine, and fluorodeoxyuridine) for 24 h near the beginning of the culture (∼3 to 7 days).

17.3.3 Organotypic SCN Slice Culture on the MED Probe

For the organotypic slice culture (Figure 17.1c), an SCN pair is dissected from a coronal hypothalamic slice 200∼250 μm thick for mice and 250∼350 μm for rats. The explants are transferred to the collagen-coated MED probe using a transfer pipette. After removing the excess medium from the probe, the SCN slice is incubated in a CO_2 incubator with 100% humidity for 1∼2 h until the slice attaches to the collagen gel. Then the slices are incubated with ∼250 μl DMEM/F12 medium supplemented with 5% fetal bovine serum for the first two days, and thereafter with no serum. Of critical importance, slices are kept wet but not submerged in medium. Within a few days, neurites extend from the periphery of the slice, and slices become thinner (Nakamura et al., 2001; Figure 17.1c). Spontaneous discharges of single SCN neurons are monitored from five days up to two months after the start of the culture.

17.3.4 Long-Term Monitoring of Spike Counts

For circadian rhythm analysis, spike numbers are monitored continuously throughout the duration of the experiment. To maintain the constant conditions this requires, half the medium is exchanged every day during recording. The number of spike discharges is counted by discriminating the spikes using a Time-Window Slicer (Nihon Kohden) and feeding them into a computer every 10 sec. The circadian periodicity ($p < 0.01$) and circadian period are computed using a chi-square periodogram (Sokolove and Bushell, 1978).

17.4 Circadian Periods of Clock Neurons in Dispersed Cell Cultures and Organotypic Slice Cultures of the SCN

SCN neurons exhibited robust circadian rhythms in their spontaneous discharges (Figure 17.2), and their periods were specific to each neuron (Welsh et al., 1995; Honma et al., 1998b). We recently compared the distributions of circadian periods in single SCN neurons between the two culture types (Honma et al., 2004). Of 308 neurons in 54 dishes examined in the dispersed cell culture, 220 neurons (71.4%) exhibited significant circadian rhythms in their firing. In the slice culture, out of 204 neurons from 24 slice cultures examined, 185 neurons (82.6%) exhibited significant circadian firing rhythms. The percentage of rhythmic neurons was significantly higher in the slice culture than in the dispersed cell culture.

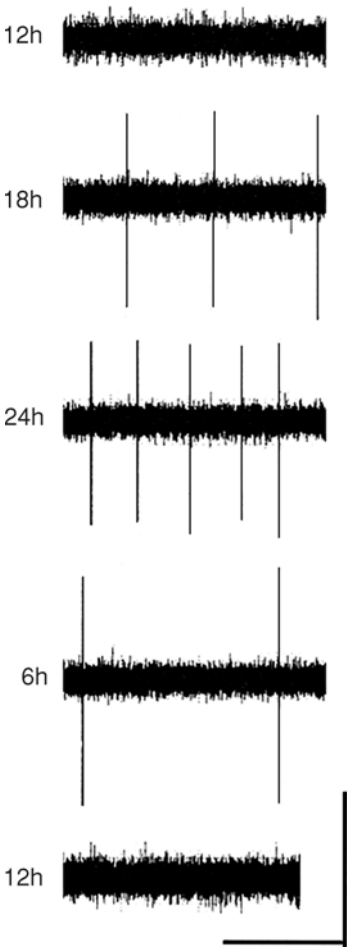

FIGURE 17.2. Spontaneous firing rhythms of a rat SCN neuron. Neuronal activity was recorded from a single SCN neuron every 6 h. Almost no firing occurs at 12:00 h, but regular firing is apparent at 24:00. Scale: 50 μV, 1 sec.

The circadian periods followed a Gaussian distribution in both cultures, and their mean circadian periods were not significantly different from each other. However, the distribution range was significantly narrower in the slice culture than in the dispersed cell culture. The circadian periods of spontaneous firing rhythms varied between 20.0 to 30.9 h with an average period of 24.1 ± 1.4 h (mean ± SD) (Figure 17.3a) in the dispersed cell culture, and between 22.4 to 26.7 h, with an average of 24.2 ± 0.7 h (Figure 17.3b) in the slice culture.

Within each dish of the dispersed cell cultures, periods of neuronal rhythms varied widely, whereas for the slice cultures, the rhythms monitored from different neurons were basically synchronized. Furthermore, in the dispersed cell cultures,

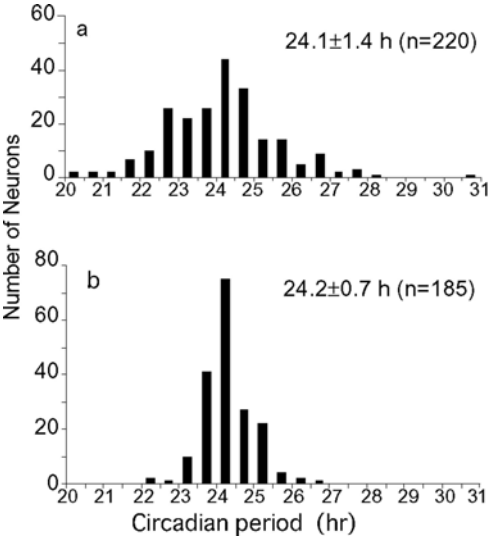

FIGURE 17.3. Diversity of circadian periods in individual SCN neurons depends on the culture method. Circadian period distribution in (a) the dispersed cell culture and (b) slice culture. The mean periods are not significantly different, but the distribution ranges differ significantly between the two cultures. (Adapted with permission from Honma et al., 1998b.)

but not in the slice cultures, there was a significant correlation between the period length and variation of circadian rhythm: the more the mean circadian period in a culture dish deviated from the overall mean, the larger the standard deviation of period in a dish became. These results suggest that in addition to the SCN cell assemblage with its close cell–cell apposition, the intrinsic circadian period plays a significant role in synchronizing the constitutional oscillators in the SCN. Furthermore, the entire SCN structure, including afferents from extra-SCN areas, seems to be necessary for the SCN neurons to express the rhythms with physiological periodicity, because the free-running periods of behavioral rhythms in rats (24.4 ± 0.2 h, $n = 147$) were distributed within a very narrow range of 24.0 and 24.8 h.

17.5 How Do Clock Neurons Talk to Each Other: Cross-Correlation Analysis to Assess Neuronal Interactions

Because the SCN is composed of many clock neurons with slightly but significantly different circadian periods, it is critical for these neurons to communicate with each other in order to synchronize and express a single circadian periodicity within the SCN. There are at least three modes of communication for SCN neurons to convey circadian information: chemical synapses, gap junctions (Shinohara et al., 2000), and diffusible factors (Silver et al., 1996). Using the dispersed SCN cell culture on the MED probe, we computed cross-correlation analyses of spike timings to

determine if synaptic coupling existed, and, if so, whether it played an excitatory or inhibitory role.

In addition, the coupling through gap-junctions was also evaluated by computing cross-correlograms. In principle if diffusible factors could couple neuronal rhythms, neurons located nearby would possess the same period, although this would not be the case in the dispersed SCN cell culture. Our data (Honma et al., 1998b; Shirakawa et al., 2000) indicated that, in fact, neuronal rhythms could not be coupled by diffusible factors except those which were degraded or diluted immediately.

We analyzed the relationship between synchronization of circadian rhythms and synaptic communication in 310 neuron pairs in the dispersed cell culture of the rat SCN (Shirakawa et al., 2000). Seventy-eight neuron pairs (24%) displayed circadian firing rhythms in both neurons, and among them 35 (45% of neuron pairs with circadian rhythms) showed synchronized circadian rhythms. In those neuron pairs with synchronized circadian rhythms, we detected either excitatory (32) or inhibitory (3) communication without exception (Figures 17.4 and 17.5). The interspike interval (ISI) between the neuron pairs exhibiting excitatory communications was 1.28 ± 0.23 msec (mean $\pm$ SE), and 25 of the 32 pairs had an ISI shorter than 2 msec. Among the neuron pairs with inhibitory communication, the neuronal firing of the postsynaptic cell was suppressed for 100 to 140 msec

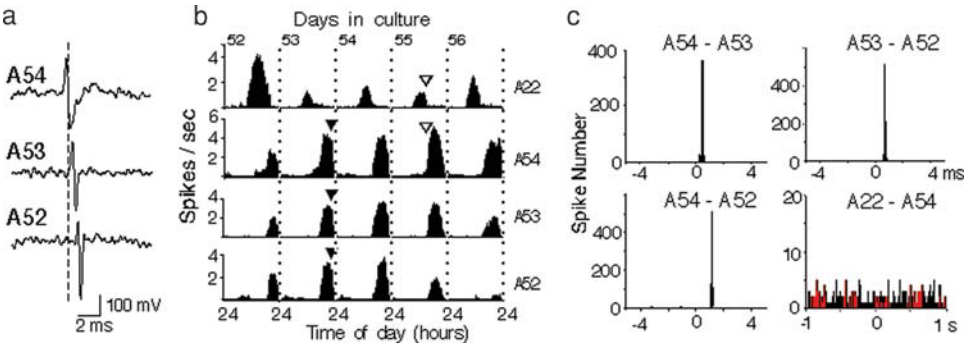

FIGURE 17.4. Excitatory synaptic communication between neuron pairs with synchronized circadian rhythm. (a) Spike waveforms of neurons A54, A53, and A52 on the same MED probe. The interpolar distances from A54 to A53 and to A52 are 150 and 300 μm, respectively. (b) Synchronization and desynchronization of circadian firing rhythms in the four neurons. The circadian rhythm is expressed as the mean firing rate (spikes/sec) in 15 min. Closed triangles indicate the time when cross-correlations were analyzed between A52, A53, and A54; and open triangles, between A22 and the other three neurons. (c) Cross-correlograms indicating excitatory synaptic communication between the neurons (A54, A53, A52) whose circadian rhythms were synchronized; and absence of functional communication between A22 and A54. Cross-correlation was not detected between A22 and A52 or A53 (data not shown). The abscissa is the timing of spikes with bins of 100 μs (except for A22 to A54) and of 10 ms (A22 to A54), and the ordinate is the cumulative number of spikes. (Adapted with permission from Shirakawa et al., 2000.)

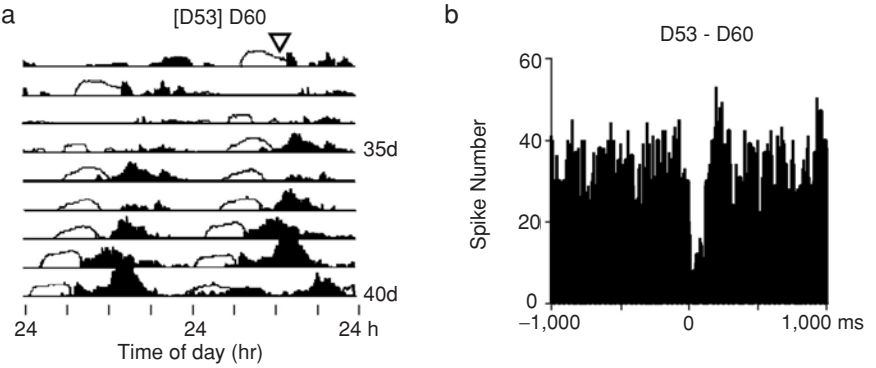

FIGURE 17.5. Inhibitory interaction between neurons showing synchronized circadian firing rhythm but with a phase-lag of about 6 h. (a) Double-plotted circadian firing rhythm of cell D53 (outlines) superimposed on that of cell D60. The circadian periods of both neurons are 23.6 hr. Cross-correlation was analyzed at the time marked by the open triangle. The numbers in the right margin indicate the number of days in cell culture. The full scale of firing rate: 8 (Hz, D53) and 6 (D60). (b) Cross-correlogram between cells D53 and D60 showing that D53 exerted a synaptic inhibition on D60. The two neurons were located on adjacent electrodes. (Adapted with permission from Shirakawa et al., 2000)

after the firing of the presynaptic cell (Figure 17.5). In 43 pairs out of 78 examined, circadian rhythms were not synchronized, and no significant correlation was detected in the timing of spikes in any of the desynchronized pairs (Figure 17.6).

These cross-correlation data strongly suggest that the circadian rhythms of SCN neurons are coupled by synaptic communication, at least in the dispersed cell

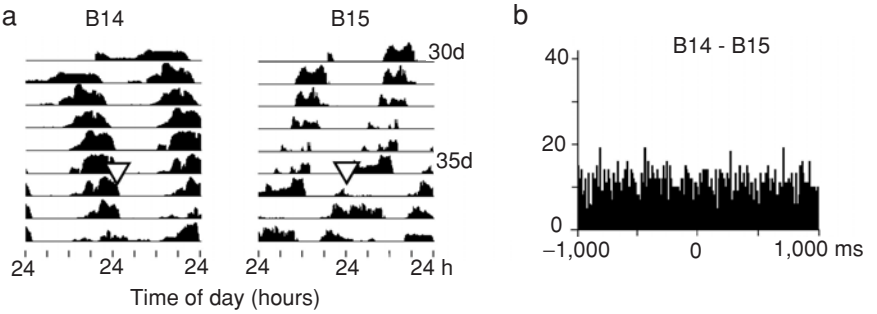

FIGURE 17.6. A lack of cross-correlation between neurons exhibiting circadian firing rhythms with different circadian periods. (a) Double-plotted circadian firing rhythms of two neurons located on adjacent electrodes. Circadian periods of cells B14 and B15 are 25.2 h and 22.8 h, respectively. Open triangles indicate the time when cross-correlation (b) was analyzed. The full scale of firing rate: 7 (B14) and 4 (B15). (b) Cross-correlogram between two neurons showing no significant correlation in spike timing. (Reproduced with permission from Shirakawa et al., 2000.)

culture. It remains unknown why only a few inhibitory communication neuronal pairs were found in spite of the fact that most SCN neurons contain gamma-aminobutyric acid (GABA), and GABA is reported to be responsible for inhibitory neurotransmission in the SCN (Moore and Speh, 1993; Strecker et al., 1997).

One possibility is that other inhibitory pairs were undetected if their constituent cell firings were not sufficiently concurrent (i.e., both occurring within the sampling/recording window used). Circadian firing rhythms so far recorded from inhibitory communication neuronal pairs were significantly out of phase as depicted in Figure 17.5a, suggesting difficulty in detecting inhibitory communication via cross-correlation analysis. Wagner et al. (1997, 2001) reported that GABA depolarized SCN neurons during the subjective day (i.e., the active phase) but hyperpolarized them during the subjective night (i.e., the inactive phase), and they attributed this to shifts in the chloride equilibrium potential. We thus examined the involvement of GABAergic communication in our SCN cell cultures. In dispersed SCN cell cultures, about 60% of the neurons were immunoreactive to antibodies against either GABA or glutamic acid decarboxylase (GAD). Bicuculline, the $GABA_A$ receptor antagonist, increased the firing rate in 36 neurons and decreased it in 7 out of 43 examined at 10 μM; and increased it in 40 neurons while decreasing it in 3 at 50 μM (Shirakawa et al., 2000). In some neurons, bicuculline dose-dependently decreased the firing rate during the active phase (Figure 17.7a), and increased it during the inactive phase (Figure 17.7b). This suggests that, at least for some SCN neurons, GABA can play either an excitatory or inhibitory role depending on the circadian phase.

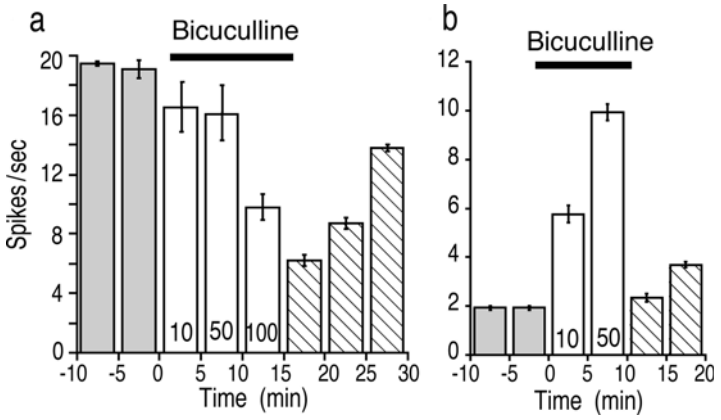

FIGURE 17.7. Effects of bicuculline on spontaneous firing activity of a single SCN neuron. A dose-dependent decrease in the firing rate during the active phase (a) or increase during the inactive phase (b) was observed in the same neuron measured 8 days apart. Numbers in the bars indicate concentrations of bicuculline (μM). Data are spike rates before (gray bars), during (open bars), and after (hatched bars) bicuculline application (mean ± SE). Bicuculline application begins at abscissa time 0. (Reproduced with permission from Shirakawa et al., 2000.)

The results of cross-correlation analysis suggest that excitatory communication contributes to the synchronization of circadian oscillators by delivering signals on the order of milliseconds, whereas inhibitory communication does so by modulating the excitability or coupling strength of the oscillating SCN neurons.

Recently Yamaguchi et al. (2003) studied cultured coronal SCN slices from transgenic mice expressing a promoter-driven luciferase reporter of the clock gene, *Period1* (*Per1*), and they established that individual SCN neurons exhibited synchronized, rhythmic *Per1* expression. When slices were subsequently dissected horizontally at the upper one-third, the dorsal third became desynchronized and the larger ventral section remained in synchrony. Their results suggest that it is not the close apposition of SCN neurons per se, but rather the intactness of larger regions—or even simply the total number of cell assemblages—that is necessary for synchronized, rhythmic gene expression. Further studies are needed to monitor simultaneously both neuronal and gene expression rhythms.

17.6 Sodium Channel-Dependent Cell Communication

Because the circadian rhythms of SCN neurons are thought to synchronize with functional synapses, we examined the effects of TTX on the coupling of the circadian firing rhythms. TTX suppressed the spontaneous firing of a single SCN neuron, but did not suppress its internal circadian oscillator (Figure 17.8a), which was consistent with the previous report of Welsh et al. (1995). We also examined the effects of TTX treatment, applied over seven consecutive days, on the synchronization of neuronal circadian firing (Honma et al., 2000). Of seven pairs whose circadian rhythms were initially synchronized, TTX induced desynchronization and loss of cross-correlation in five of them. In the remaining two pairs, rhythms maintained synchrony, but their ISIs were elongated.

In short, suppressing synaptic communication gradually desynchronized circadian firing rhythms of individual neurons. The effects of TTX were also examined in organotypic SCN slice cultures, where most neuronal rhythms were already synchronized. After the washing out of TTX, neuronal firing recovered in all slices examined; however, in most slices, circadian firing rhythms were desynchronized or had altered periods (Honma et al., 2002; Figure 17.8b). These results of TTX treatment suggest that the synaptic coupling of circadian rhythms is dynamic and may be activity-dependent.

17.7 Circadian Firing Rhythm in the SCN in Behaviorally Arrhythmic Clock Mutant Mice

Cell and tissue cultures on the MED probes provide excellent tools to examine a particular gene function. By culturing fetal or newborn brain tissues, neuronal activity can be monitored for long extents, even when obtained from mutant or

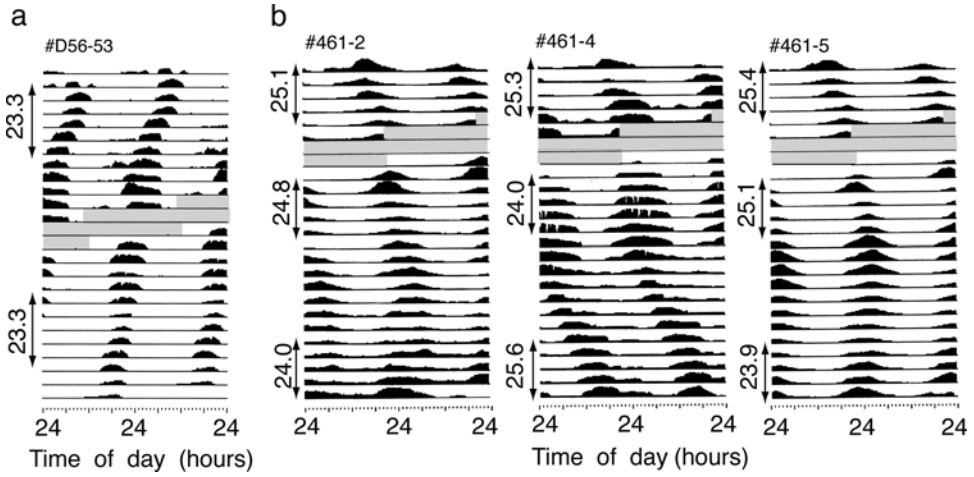

FIGURE 17.8. Effects of tetrodotoxin (TTX) on the circadian firing rhythms of a dispersed SCN neuron (a) and a slice culture (b). (a) Double-plotted actogram showing the circadian firing rhythm of a single SCN neuron in a dispersed cell culture before and after the application of 500 μM of TTX for 2 days (shadowed areas in the actogram). The number at the top of each actogram is the neuron identifier. Numbers along the left margin indicate the circadian periods over the days spanned by arrows. (b) Double-plotted circadian firing rhythms of 3 neurons from a single SCN slice. Before the TTX treatment (200 μM), 8 neurons on a probe expressed synchronized circadian firing rhythms which gradually desynchronized after treatment. (Honma et al., 2002, Society for Neuroscience Meeting.)

gene knockout animals with lethal defects. In addition, modifying gene expression via adenovirus vectors, antisense oligonucleotides, or siRNA is much easier with a culture system than with whole animals. By culturing tissue on the MED probes, we could examine gene functions while simultaneously monitoring neuronal activity.

We studied the role of the clock gene, *Clock*, by measuring spontaneous firing rhythms in the SCN of *Clock* mutant mice (Nakamura, et al., 2002). Under constant darkness (DD), behavioral rhythms of heterozygotes (*Clock/+*) free-ran with periods longer than wild-types, and those of homozygotes (*Clock/Clock*) became arrhythmic (Vitaterna, 1994; Figure 17.9d–f). *Clock*, which codes a basic helix–loop–helix transcription factor, is regarded as a crucial component of the molecular feedback loop generating the circadian rhythm intracellularly (Gekakis, 1998). In organotypic slice cultures of *Clock* mice SCNs, significant circadian rhythms were detected in 95% of neurons recorded from wild-types, 83% from *Clock/+* and 77% from *Clock/Clock*. The maximum firing rate differed from neuron to neuron but was remarkably constant for any given neuron, and the amplitude of firing rhythms did not vary among genotypes either (Figure 17.9g–i). The mean circadian period was 23.5 h in wild type, 24.8 h in *Clock/+*, and 27.2 h in *Clock/Clock*, respectively. The periods of wild-type and *Clock/+* corresponded

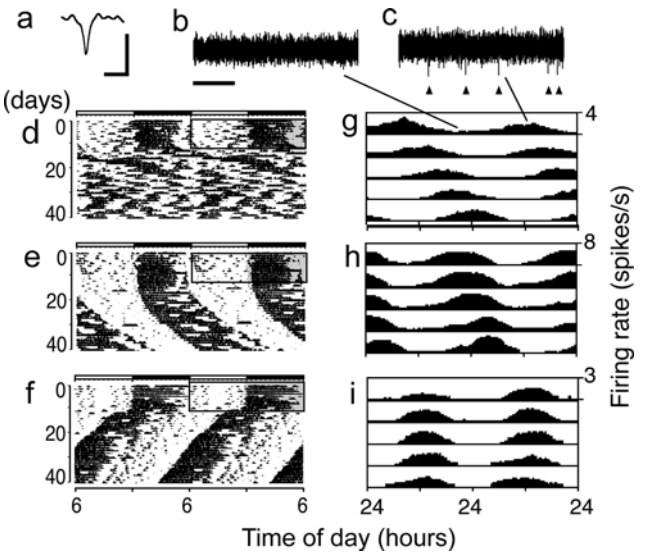

FIGURE 17.9. Significant circadian rhythms in neuronal firing contrasts with arrhythmic wheel-running rhythms in *Clock* mutant mice. (a) Averaged waveform of ten spikes from a *Clock/Clock* neuron. Scale: 100 μV, 2 ms. (b)–(c) Raw voltage trace of a *Clock/Clock* neuron recorded (b) during rest time, and (c) during activity time as indicated in actogram. Triangles indicate discriminated spikes. (d)–(f) Wheel-running activity rhythm of (d) *Clock/Clock*, (e)*Clock/+*, and (f) wild-type mice. White and black bars above each actogram, and open and shaded squares within each actogram, designate the light and dark phases of the 12 h light and 12 h dark cycle, respectively. After 10 days of recording under the light–dark cycle, mice were released into complete darkness. (g)–(i) Histograms of circadian firing rates of (g) *Clock/Clock*), (h) *Clock/+*, and (i) wild-type mice. Numbers in the top right corner of each panel indicate the scales of firing rates. (Reproduced with permission from Nakamura et al., 2002.)

well with that of behavioral rhythms in each genotype. On the other hand, SCN neurons of behaviorally arrhythmic *Clock/Clock* mice exhibited robust firing rhythms.

The findings indicate that the *Clock* mutation lengthens the circadian period but does not abolish the circadian oscillation itself. Surprisingly, in the dispersed cell culture, only 15% of neurons exhibited circadian firing rhythms in *Clock/Clock* SCNs, and their periods were lengthened. By contrast this was observed in 46% of wild-type SCNs. In short, the percentage of neurons with circadian rhythms was affected by both culture method and genotype. The results also suggest that the number of clock neurons is much less than had been previously expected. Indeed, the behavioral arrhythmicity observed in *Clock/Clock* mice might be attributable to these two findings involving clock neurons: their relative scarcity and their extremely long circadian periods. Such a combination could result in weakened intercellular communication and synchronization between SCN neurons.

17.8 Perspectives

By culturing SCN neurons on the MED probe, we have examined the properties of the circadian firing rhythms and the mechanisms with which they talk to one another. Autoregulatory transcription and translation feedback loops involving several clock genes and their products are proposed as a molecular machinery of the circadian rhythm generation (Reppert and Weaver, 2001). However, it remains basically unknown how the molecular oscillation is transmitted to cellular functions such as neuronal discharges (Honma and Honma, 2003). Recently Ikeda et al. (2003) simultaneously measured intracellular Ca^{++} levels and neuronal discharges by culturing SCN slices transfected with the Ca^{++}-binding fluoroprotein Cameleon on the MED probe and suggested that Ca^{++} is one of the key substances which links the molecular clock to cellular functions.

Most peripheral tissues express robust circadian rhythms in *Clock* gene expression in vitro, yet in vivo SCN lesioning results in the total loss of circadian rhythms in both behavior and physiology. The discrepancy suggests that the SCN serves to coordinate peripheral clocks via both neural and humoral mediators, but the mechanisms for this also remain mostly undetermined (Yoo et al., 2004).

How the SCN functions as the body's pacemaker still remains a mystery, but our goal of solving it is within reach, for we have now in hand excellent leading-edge tools at our disposal, including bioluminescent and fluorescent reporter genes, and, of course, the MED probe.

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