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Individual Neurons Dissociated from Rat Suprachiasmatic Nucleus Express Independently Phased Circadian Firing Rhythms

David K. Welsh,*† Diomedes E. Logothetis,‡‖ Markus Meister,†§ and Steven M. Reppert*†
*Laboratory of Developmental Chronobiology
Massachusetts General Hospital
Boston, Massachusetts 02114
†Program in Neuroscience
Harvard Medical School
Boston, Massachusetts 02115
‡Division of Cardiology
Children's Hospital
Boston, Massachusetts 02115
§Department of Molecular and Cellular Biology
Harvard University
Cambridge, Massachusetts 02138

Summary

Within the mammalian hypothalamus, the suprachiasmatic nucleus (SCN) contains a circadian clock for timing of diverse neuronal, endocrine, and behavioral rhythms. By culturing cells from neonatal rat SCN on fixed microelectrode arrays, we have been able to record spontaneous action potentials from individual SCN neurons for days or weeks, revealing prominent circadian rhythms in firing rate. Despite abundant functional synapses, circadian rhythms expressed by neurons in the same culture are not synchronized. After reversible blockade of neuronal firing lasting 2.5 days, circadian firing rhythms re-emerge with unaltered phases. These data suggest that the SCN contains a large population of autonomous, single-cell circadian oscillators, and that synapses formed in vitro are neither necessary for operation of these oscillators nor sufficient for synchronizing them.

Introduction

Circadian clocks are endogenous biological oscillators that orchestrate daily patterns of many physiological and behavioral processes. In the absence of a light–dark cycle, circadian clocks cause these daily patterns to persist as "circadian" (circa daily) rhythms, with periods near 24 hr. The principal mammalian circadian clock is located in the suprachiasmatic nucleus (SCN), a paired neuronal structure at the base of the hypothalamus, just above the optic chiasm (Klein et al., 1991).

Spontaneous circadian rhythms in neuronal firing rate can be recorded from populations of SCN neurons, either as multiple-unit recordings (Inouye and Kawamura, 1979; Bouskila and Dudek, 1993) or as ensemble averages of many brief single-unit recordings (Green and Gillette, 1982; Groos and Hendriks, 1982; Shibata et al., 1982). The existence of such population rhythms, in vivo or in slice preparations, implies that there must be communication of circadian phase information among SCN neurons, allowing them to oscillate in synchrony. This is also supported by direct evidence that circadian oscillations within the SCN are synchronous among different subregions (Schwartz et al., 1980; Zlomanczuk et al., 1991).

The precise role of intercellular communication within the SCN is uncertain. One theoretical possibility is that the SCN is a multicellular network generating circadian oscillations as an emergent property, much as locomotor rhythms in invertebrates are generated by neuronal circuits with specific patterns of connections (Selverston and Moulins, 1985). This began to seem unlikely, however, after demonstrations that populations of SCN cells retain the capacity for circadian oscillation after being dissociated into single cells (Silver et al., 1990; Murakami et al., 1991; Watanabe et al., 1993). Furthermore, in the mollusk Bulla gouldiana, there is evidence that single, isolated neurons can generate circadian rhythms (Michel et al., 1993). Thus, it is quite possible that intercellular communication in the SCN is not part of the circadian clock mechanism itself, but rather acts to synchronize a distributed population of single-cell circadian oscillators. The SCN, therefore, could provide a unique opportunity to study a pervasive aspect of mammalian physiology and behavior at the level of a single cell.

Which types of SCN cells might be capable of acting as circadian oscillators is presently unknown. The SCN consists of a heterogeneous population of neuronal and glial cells. Most or perhaps all SCN neurons are GABAergic (Okamura et al., 1989; Decavel and van den Pol, 1990; Moore and Speh, 1993), but subtypes can be distinguished by differential expression of neuropeptides, anatomical connections, and responsiveness to neurotransmitters (van den Pol and Tsujimoto, 1985; Watts and Swanson, 1987; Meijer and Rietveld, 1989). SCN astrocytes may also be heterogeneous, as evidenced by varied responses to neurotransmitters (van den Pol et al., 1992). How do these various cell types contribute to circadian clock function, and how do they communicate with one another?

Cellular analysis of circadian oscillation in the SCN has so far been hampered by the lack of an experimental system providing both single-cell resolution and long-term stability. Here we demonstrate automated, long-term monitoring of circadian firing rhythms from multiple individual SCN neurons in parallel, in a dissociated culture system in which cell interactions can be manipulated and individual rhythmic cells are accessible for further detailed study. Despite abundant functional synapses, SCN neurons in the same culture expressed circadian rhythms of widely different phases, and even different circadian periods. The rhythms re-emerged unperturbed after reversible blockade of neuronal firing lasting 2.5 days. These data provide strong evidence that the SCN is composed of multiple circadian oscillators.

‖Present address: Department of Physiology and Biophysics, Mt. Sinai Medical Center, Box 1218, 1 Gustave L. Levy Place, New York, New York 10029-6574.

Table 1. Immunolabeling of Neurons in SCN Cultures

Antigen	Percentage of Neurons Immunopositive
γ-aminobutyric acid (GABA)	69.4 ± 1.9
Vasopressin neurophysin (VP–NP)	13.5 ± 0.7
Vasoactive intestinal peptide (VIP)	8.7 ± 0.5
Somatostatin (SS)	1.0 ± 0.1

Values are means ± SEM of three determinations made from separate dissociations.

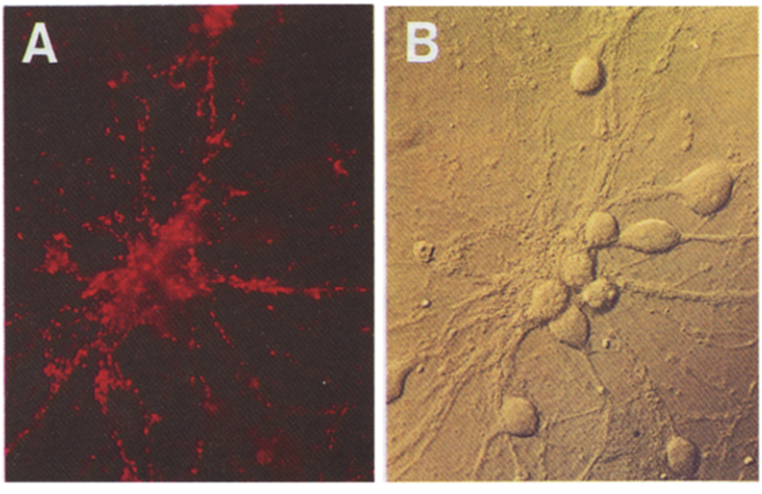
Results and Discussion

Characterization of Dissociated SCN Cells

Cells dissociated from neonatal rat SCN were characterized after 2 weeks in culture, by morphology and by immunolabeling. SCN neurons had long processes (typically >100 μm), relatively simple dendritic arbors, and compact cell bodies (diameter = 10.0 ± 2.1 μm, mean ± SD, shortest axis; n = 33). Many cell bodies were spindle-shaped, but others were round, polygonal, pyramidal, or pear-shaped. Neurons rested on a layer of astrocytes, identified by immunolabeling for glial fibrillary acidic protein (GFAP). Astrocytes were initially rare but multiplied and flattened progressively with time in culture, achieving confluence after 1–2 weeks.

Neurons at 2 weeks in culture were further characterized by immunolabeling for γ-aminobutyric acid (GABA), and for the neuropeptides vasopressin (VP), vasoactive intestinal peptide (VIP), and somatostatin (SS). These peptides delineate distinct neuronal subpopulations of the predominantly GABAergic SCN (Dierickx and Vandesande, 1979; Watts and Swanson, 1987; Moore and Speh, 1993). The only correlation observed between neuropeptide phenotype and cell morphology was that many SS cells were pyramidal (41%; n = 68). Relative proportions of GABA, VP, VIP, and SS cells (Table 1) were very similar to those found in the SCN in vivo, indicating that representative subsets of SCN neurons remained viable in culture.

Percentages of VP and VIP cells were only about half of those found in vivo, however, and the percentage of GABA cells was also somewhat lower (Moore and Speh, 1993). Selective mortality of VP and VIP cells with time in culture is unlikely, as similarly low numbers were obtained even at 1 day in culture. It is possible that some VP and VIP cells may have escaped detection in vitro, owing to weaker neuropeptide expression, or because cells were



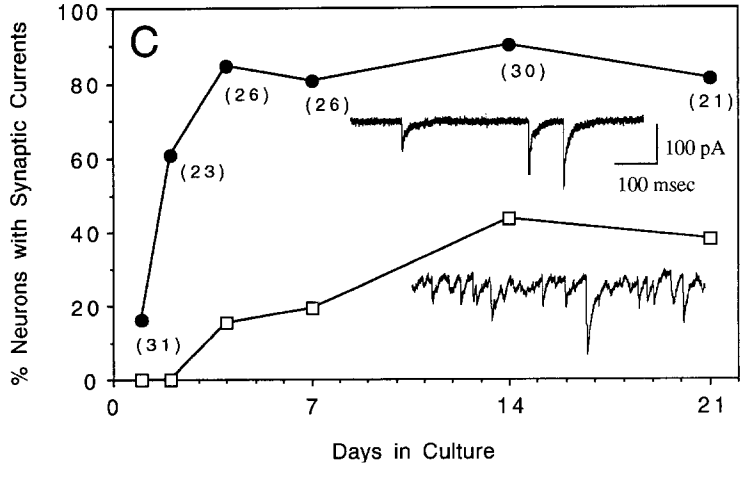


Figure 1. Prevalence of Synapses in SCN Cultures

(A) SCN neurons cultured for 3 weeks and labeled with antibody to SV2, a protein specific for synaptic vesicles. Synapses appear as dots of red immunofluorescence bordering neuronal somata and dendrites. Magnification, 500×.

(B) Same field viewed by differential interference contrast optics.

(C) Percentages of neurons exhibiting at least one synaptic current (circles) or continuously overlapping synaptic currents (squares) at various times in culture, determined by whole-cell patch recordings of 1 min duration. Numbers of cells recorded at each time point are indicated in parentheses. Examples of synaptic activity in each category are shown in insets (standard intracellular solution; membrane potential = −60 mV). Synaptic currents were recognized by rapid onset and slower exponential decay over tens of milliseconds.

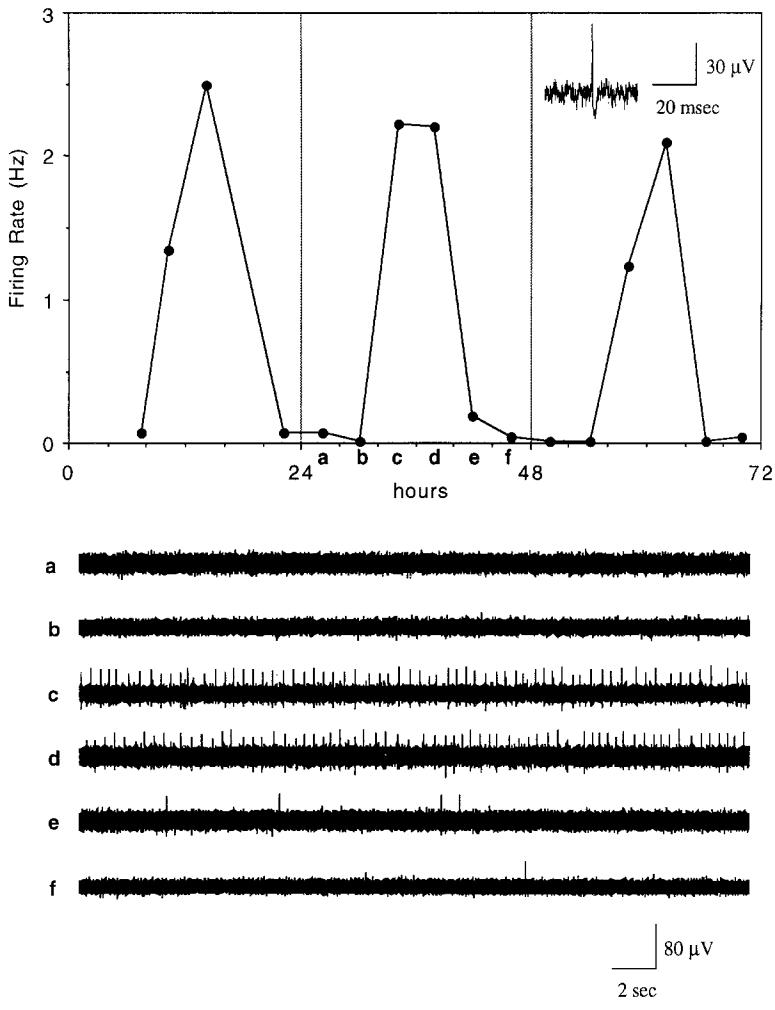


Figure 2. Circadian Firing Rhythm of an Individual SCN Neuron

Circadian firing rhythm of a neuron cultured for 1 week and recorded for 3 days at about 4 hr intervals, using the multielectrode plate (MEP) technique. Each point represents the mean firing rate for a 5 min record. A brief raw voltage trace (inset) shows the typical biphasic waveform of an action potential (a spike). Negative extracellular voltage is plotted upward. Shown below are longer raw voltage traces recorded at the time points labeled a–f, illustrating the circadian variation in firing rate.

not pretreated with colchicine. But, because VP, VIP, and GABA cells are all rarer outside the SCN, it is also quite possible that the smaller numbers found in vitro were largely due to imperfections in dissection. Thus, as many as half of all the cultured neurons may have originated from outside the anatomical boundaries of the SCN.

Synaptogenesis In Vitro

Prevalence of synaptic connections was assessed after various times in culture (1, 2, and 4 days; 1, 2, and 3 weeks), by both anatomical and electrophysiological methods. Synapses were identified anatomically by immunolabeling for SV2, a protein specific to synaptic vesicles (Buckley and Kelly, 1985). Synapses first appeared within 1 day and were plentiful by 3 weeks in culture, appearing as dots of SV2 immunofluorescence (Figure 1A) located along the borders of neuronal somata and dendrites (Figure 1B). In a complementary functional approach, synaptic activity was detected by whole-cell patch recording of spontaneous synaptic currents (Figure 1C). Electrophysiology confirmed that most cells acquired their first synaptic input within a few days in vitro, and that synapse number increased to substantial levels by the third week.

Synaptic currents reversed direction near the calculated Nernst potential for Cl^- and were blocked by bath application of 50 μM bicuculline (n = 2), which are distinguishing features of inhibitory $GABA_A$ currents. No excitatory synaptic currents were observed. Most of these postsynaptic currents were probably driven by presynaptic action potentials, as they were much larger and more frequent than the miniature GABA currents ("minis") that persist in other culture systems when both Na^+ and Ca^{2+} action potentials are blocked (Kraszewski and Grantyn, 1992).

Development of Spontaneous Neuronal Firing

Spontaneous firing behavior of neurons in SCN cultures was examined initially by conventional, short-term, cell-attached patch (CAP) recording, after various times in culture (1, 2, and 4 days; 1, 2, and 3 weeks). Overall, spikes were generated by 49% of recorded neurons (n = 594), consistent with results from a previous study (Walsh et al., 1992). The proportion of active neurons increased with time in culture ($p < .001$), from 31% at 1 day to a maximum of 64% at 2 weeks. Of active neurons recorded for a full 5 min (n = 171), spike rates ranged from 0.03 to 9.9 Hz (mean = 2.5 Hz). Mean firing rate was not related to time in culture or time of day. The majority of cells fired in an irregular or episodic pattern, but 5% exhibited a bursting

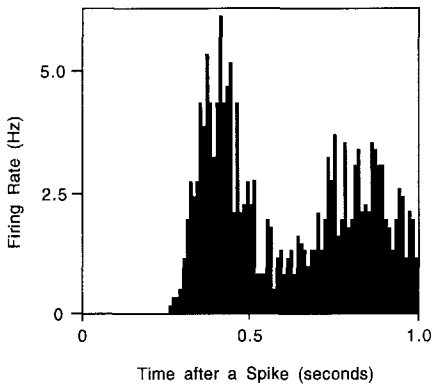


Figure 3. Firing Pattern Analysis for an SCN Neuron

The firing pattern of the SCN neuron shown in Figure 2 was analyzed in this autocorrelogram, which plots firing rate as a function of time after a spike, averaged for all spikes in a 5 min record. The firing peak, following an initial 0.25 s silent period, reflects the regularity of the cell's short-term firing pattern at a time near the maximum of its circadian firing rhythm. This is a feature typical of clock cells.

pattern and 8% fired regularly (defined as interspike interval coefficient of variation < 0.5). The proportion of regular cells increased from 2% in the first week to 25% at 3 weeks ($p = .01$).

In many cases ($n = 185$), CAP recordings were followed by immunolabeling for the neuropeptides VP and VIP. For these experiments, cells were cultured on coverslips containing labeled, photoetched grids (Bellco), to facilitate location of recorded cells after immunolabeling. No correlation was found, however, between firing rate or pattern and neuropeptide phenotype. Similarly, firing behavior was unrelated to cell size or shape, or to the number of processes.

Individual SCN Neurons Express Circadian Firing Rhythms

Circadian rhythmicity in neuronal firing rate was monitored by culturing SCN cells on special glass multielectrode plates (MEPs), each containing a fixed hexagonal array of 61 microelectrodes. These or similar plates have previously been used to record extracellular action potentials from mouse spinal neurons (Droge et al., 1986), Aplysia neurons (Regehr et al., 1989), and the isolated vertebrate retina (Meister et al., 1991). Spontaneous action potentials were recorded from more than 50 individual SCN neurons, from six separate dissociations. Clear circadian rhythms in firing rate (Figure 2) were consistently observed in about half of the cells recorded in each culture. Circadian rhythms were observed as early as 1 week and as late as 7 weeks after dissociation (the earliest and latest times examined). Of SCN neurons recorded at 15 min intervals for at least 3 days, 17 out of 32 cells (53%) exhibited statistically significant circadian rhythms ($p < .01$), with periods near 24 hr (24.35 ± 1.20 hr, mean ± SD; range 21.25–26.25 hr). Circadian rhythms were not observed in control recordings of hippocampal cells ($n = 18$; data not shown).

SCN neurons expressing circadian firing rhythms ("clock cells") generally had very regular short-term firing patterns, especially at times near their circadian peaks (Figure 2; Figure 3; interspike interval coefficient of variation = 0.56 ± 0.17, mean ± SD; $n = 17$). Peak firing rates were typically between 2 and 10 Hz. As a cell's firing rate declined from its circadian maximum, firing gradually became less regular (Kim and Dudek, 1993). Sometimes, at low firing rates, clock cells expressed widely spaced bursts of spikes (separated by tens of seconds). Many clock cells were virtually silent at the low points of their circadian firing rhythms. Thus, the same clock cell, as its firing rate varied with circadian phase, could express different short-term firing patterns, all similar to those reported for cells in SCN slices (Shibata et al., 1984).

Although clock cells appeared to be more abundant than VP, VIP, or SS cells, only spontaneously active cells were studied by MEP recording, introducing the possibility of sampling bias in favor of one or another neuropeptide subtype. Indeed, in CAP recordings, only 54% of SCN neurons were active at 1–3 weeks in culture ($n = 390$). Immunolabeling after CAP recordings ($n = 185$), however, revealed that VP and VIP cells were not overrepresented among active cells; that is, VP and VIP cells were no more common among active cells than among silent cells. Thus, the fact that clock cells were so abundant (about 50% of active cells) indicates that circadian oscillations could not have been restricted to VP, VIP, or SS cells, which altogether accounted for only 23% of the neurons in these cultures (Table 1).

SCN Neurons in the Same Culture Express Independent Circadian Firing Rhythms

Simultaneous recordings of multiple cells revealed that the phases of circadian firing rhythms varied widely among cells in the same culture (Figure 4). In fact, there was no clustering of phases and no consistent relationship between cell proximity and similarity of circadian phase. In Figure 4, for example, the only 2 cells that were similarly phased (A5 and G3b) were recorded from electrodes located at opposite edges of the array (Figure 4B). In another case, 2 closely adjacent cells (recorded from the same electrode and discriminated by spike amplitude) expressed rhythms of opposite phase, their peaks occurring nearly 12 hr apart (Figure 5). Neither similarly phased nor oppositely phased cell pairs were particularly common, however. Statistical analysis confirmed that the phase differences between clock cells were uniformly distributed between 0 and 12 hr (1 hr bins; $n = 42$ pairs; $\chi^2 = 14.6$; $p > .10$), and that there was no correlation between phase difference and distance between recording electrodes ($r = .02$).

One explanation for these results is that different clock cells were expressing independent circadian oscillations with different periods. Two of cells expressing rhythms of even slightly different periods should exhibit a continuously varying phase relationship if monitored for a very long time. In fact, long-term recordings lasting several weeks revealed that cells in the same culture could indeed express distinct circadian periods (Figure 6A). Thus, independent circadian clocks running at different periods likely gave rise to the wide assortment of phase relationships

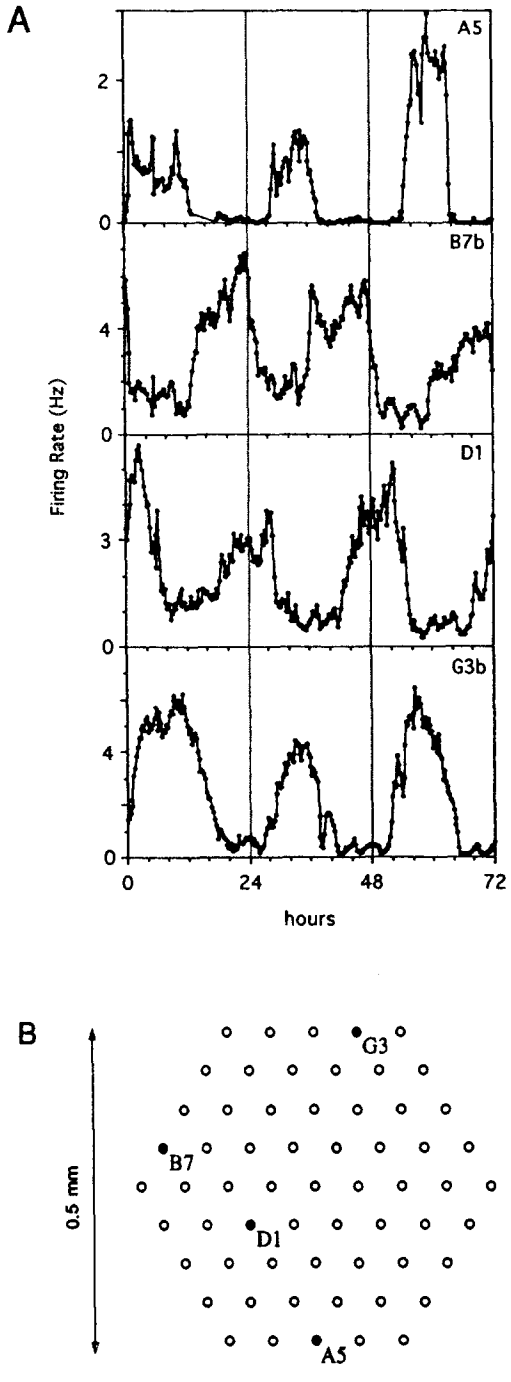


Figure 4. Differently Phased Circadian Rhythms of SCN Neurons in One Culture

(A) Neurons (4) from a single culture were recorded simultaneously, at 15 min intervals, after 1 month in culture. Each point reflects the mean firing rate for a 5 min record. Distances between recording electrodes were 185–490 μm. The first 3 cells (A5, B7b, and D1) exhibited clearly different circadian phases. In this case, 2 cells happened to be similarly phased (A5 and G3b), though they were recorded from electrodes that were the furthest apart (490 μm).

(B) Scale diagram of the electrode array, showing positions (closed circles) and names of the 4 electrodes from which these 4 cells were recorded. Positions of other electrodes are shown by open circles. Cell names correspond to electrode names, with a lower-case letter appended to distinguish multiple cells recorded from the same electrode. Electrodes were named according to a previously established convention (Regehr et al., 1989), in which an upper-case letter (A–H) denotes the amplifier to which a given electrode is connected and a numeral (1–8) denotes the region of the array where the electrode is located.

observed among clock cells in shorter experiments (Figure 4; Figure 5).

Even when 2 clock cells happened to be firing at the same time of day, their short-term firing behavior was not correlated (Figure 7A). More explicitly, cross-correlograms of spikes from pairs of clock cells (n = 42 pairs) never showed peaks or dips with time lags up to 10 s, indicating that firing of any 1 clock cell was not appreciably influenced by the firing of another (Perkel et al., 1967; Wong et al., 1993). This type of analysis rules out not only excitatory synaptic interactions, which would not be expected from the predominantly GABAergic phenotype of SCN cells, but also inhibitory synaptic interactions. Thus, despite the prevalence of inhibitory synaptic currents in whole-cell patch recordings (Figure 1C), the firing of clock cells was not detectably altered by inhibitory synaptic input. Subtle influence of combined input from many cells, however, cannot be excluded in these pairwise analyses. Importantly, both excitatory and inhibitory synaptic interactions *were* occasionally evident in cases involving nonclock cells (1 of each type, out of 130 pairs; Figures 7B and 7C), demonstrating that such interactions would have been detected if they had been present between clock cells. Excitatory interactions were also commonly detected in hippocampal cultures. Finally, this type of correlation analysis also rules out strong electrotonic coupling between SCN clock cells, which would have been evident as peaks in cross-correlograms with very short delays (<1 ms). In summary, short-term firing patterns showed no evidence of interactions among clock cells.

Circadian Rhythms Resume with Unaltered Phases after Action Potential Blockade

Circadian clocks in SCN cultures continued to operate in the absence of neuronal firing. In several experiments, action potentials were reversibly blocked for 2.5 days by bath application of the Na^+ channel blocker tetrodotoxin (TTX; 300 nM; Figure 6). No action potentials were observed in the presence of TTX. Upon removal of the drug, circadian rhythms in firing rate re-emerged, their phases consistent with projections from pretreatment rhythms, suggesting that the clocks had been running normally during TTX treatment (n = 5 trials in 4 different cells). This result, previously shown for populations of SCN neurons in vivo (Schwartz et al., 1987) or in slices (Shibata and Moore, 1993), demonstrates that the neuronal firing used here as an index of circadian function is not an essential part of the clock mechanism itself. Moreover, since conventional synaptic transmission requires invasion of action potentials into synaptic terminals, it follows that this type of communication between SCN neurons is not necessary for circadian clock function.

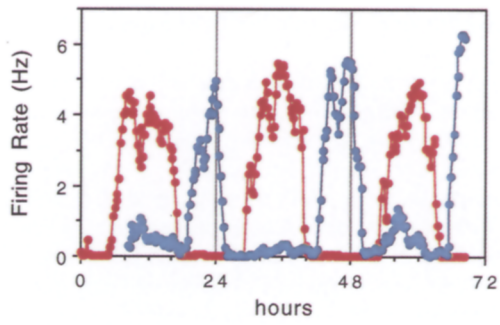


Figure 5. Oppositely Phased Circadian Rhythms of 2 Adjacent SCN Neurons

Simultaneous recordings of 2 SCN neurons in the same culture (C7a, red; C7b, blue) that were known to be very close together, because they were recorded from the same electrode and discriminated on the basis of spike amplitude. Each point reflects the mean firing rate for a 5 min record. Despite their proximity, these 2 cells exhibited circadian firing rhythms of nearly opposite phase, their peaks occurring nearly 12 hr apart. Minor peaks in the curve for cell C7b that coincide with the circadian peaks of cell C7a are artifactual, owing to partial overlap between the amplitudes of spikes from the 2 cells.

Implications for Multiplicity of Circadian Clocks within the SCN

The data presented here provide the strongest evidence to date that single SCN neurons are circadian clocks. Multiple circadian oscillations were evident within very small areas in SCN cultures, areas containing only a few hundred cells. In one extreme case, 2 adjacent neurons expressed circadian rhythms of nearly opposite phase. Furthermore, circadian firing rhythms re-emerged with unaltered phases after TTX treatment, implying that circadian clocks can operate without intercellular communication involving action potentials. It remains possible that circadian oscillation requires a small network of cells, communicating by some mechanism other than action potentials and conventional synaptic transmission (Miller, 1993; van den Pol and Dudek, 1993). Nevertheless, the simplest interpretation of our data is that individual SCN neurons expressing circadian rhythms (clock cells) are, in fact, autonomous, single-cell circadian oscillators.

The high proportion of such clock cells in our cultures (about 50%) implies that the SCN in vivo must contain a

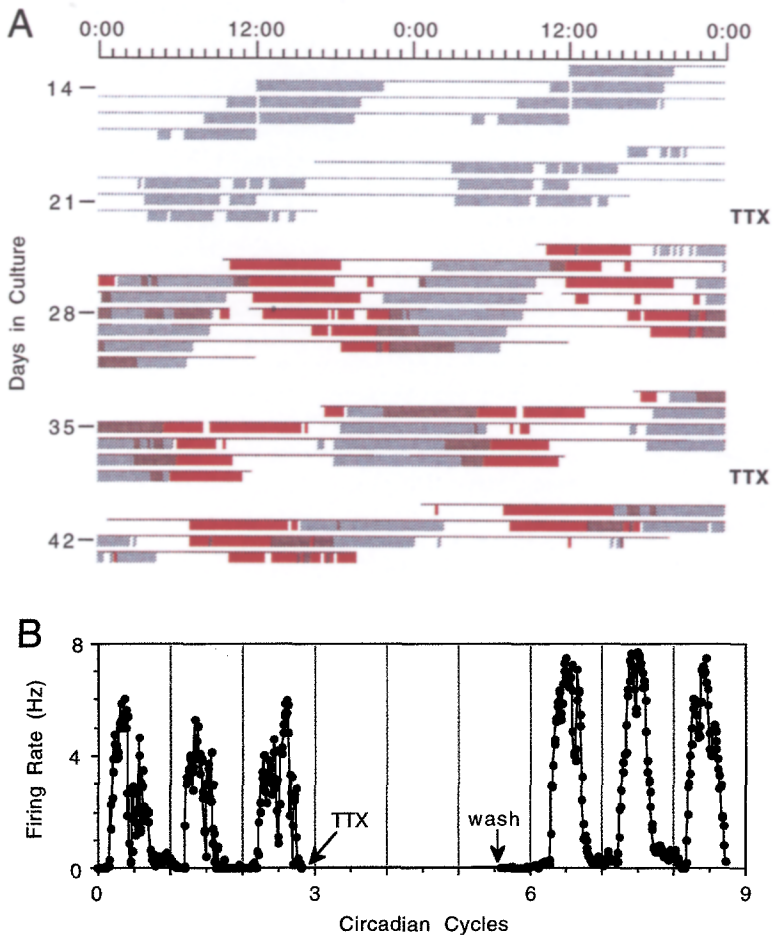


Figure 6. Long-Term Recordings and TTX Experiments

(A) Circadian firing rhythms of 2 SCN neurons in the same culture (C7a, blue; A5, red), recorded at 15 min intervals for several weeks. Successive days are plotted top to bottom, with time of day plotted left to right. Each row is extended to 48 hr, duplicating data in the next row, so that patterns crossing midnight can be appreciated. Thick bars (blue or red) show times at which firing rate was above the mean for each row. Cell C7a (blue) had a circadian period of 23.25 hr, so its rhythm drifted progressively earlier with respect to the 24 hr day. Cell A5 (red) had a period of 25.5 hr, so its rhythm drifted later. These 2 cells were recorded from electrodes 350 μm apart. Absence of a thin line indicates a gap in data collection. The second and fourth gaps in the record were experiments in which all action potentials were reversibly blocked for 2.5 days by bath application of 300 nM TTX. Circadian phases were unperturbed by these treatments.

(B) Circadian firing rhythm of cell C7a before and after TTX, showing that the recovered rhythm is in phase with the pretreatment rhythm. The horizontal time axis is marked in multiples of the cell's circadian period length (23.25 hr).

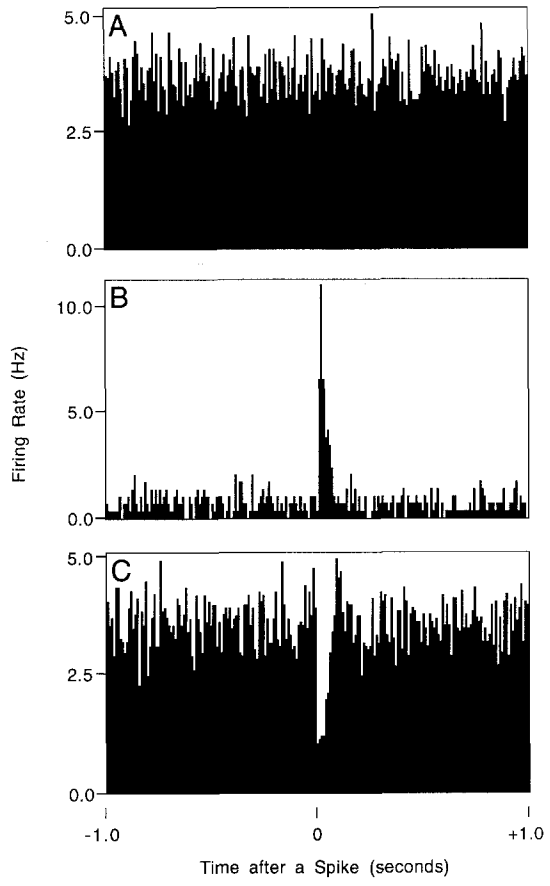


Figure 7. Firing Correlations between Pairs of Neurons in SCN Cultures

Cross-correlograms show firing rate of 1 cell as a function of time before (−) or after (+) a spike from another cell, averaged for all spikes in a 5 min record.

(A) Cross-correlogram for 2 clock cells recorded from adjacent electrodes and expressing circadian firing rhythms of similar phase. The flat curve indicates that the firing of 1 clock cell did not appreciably influence the firing of the other. This pattern was observed in all pairwise analyses of clock cells.

(B) Cross-correlogram for 1 clock cell and 1 nonclock cell in an SCN culture. The sharp peak observed at a lag time of about 25 ms indicates that the clock cell tended to fire 25 ms after the nonclock cell, a pattern consistent with excitatory synaptic interaction.

(C) Cross-correlogram for 2 nonclock cells in an SCN culture. The sharp dip observed at a lag time of about 20 ms indicates that 1 cell tended *not* to fire 20 ms after the other cell fired, a pattern consistent with inhibitory synaptic interaction.

relatively large number of potentially independent circadian oscillators. For instance, the capacity for generating circadian oscillations cannot be restricted to cells expressing the neuropeptides VP, VIP, or SS, because these altogether accounted for only 23% of neurons in the cultures. Furthermore, the neurons in our cultures included only about half as many VP and VIP cells as are found in vivo (Moore and Speh, 1993), suggesting that as many as half of all the neurons may have originated from outside the anatomical borders of the SCN. Thus, our data are consistent with the possibility that all the nonclock cells in our cultures were from outside the SCN, and that all SCN neurons are clock cells. Further characterization of clock cells should be possible by patch recording and immunolabeling of cells that express circadian rhythms during MEP recordings.

Implications for Functional Coupling of Circadian Clocks within the SCN

Circadian firing rhythms of individual SCN neurons in the same culture were not synchronized, despite abundant functional synapses. Thus, not only is conventional synaptic transmission unnecessary for circadian clock function, it is also insufficient to synchronize the circadian oscillations of dissociated SCN neurons in vitro. Previous reports of circadian firing rhythms in individual SCN neurons did not address this issue, as they were limited to 1 cell per cultured slice (Bos and Mirmiran, 1990; Khalsa et al., 1994, Soc. Neurosci., abstract). Our result does contrast, however, with the synchronization implied by previous reports of VP rhythms from dissociated SCN cultures, in which clear circadian rhythms were sometimes observed in aggregate neurosecretory output of thousands of cells (Murakami et al., 1991; Watanabe et al., 1993). Different culture conditions presumably account for the discrepancy, which perhaps could be exploited in future studies on mechanisms of synchronization.

This result also leads to the question of whether synapses are responsible for synchronizing circadian clock cells in vivo. Synapses formed in vitro might have failed to synchronize clock cells because they were somehow inferior to those formed in vivo, either in prevalence, pattern, or neurochemical content. However, SCN cells in vivo express synchronous circadian oscillations early in development (Reppert and Schwartz, 1984), before any significant synaptogenesis has occurred (Moore and Bernstein, 1989). There is also evidence for a nonsynaptic mechanism of firing synchrony among SCN neurons in slice preparations (Bouskila and Dudek, 1993). Thus, the mechanism that normally synchronizes circadian oscillations of SCN cells in vivo, and which was evidently missing from our dissociated culture system, may well be nonsynaptic (Miller, 1993; van den Pol and Dudek, 1993).

Among potential nonsynaptic mechanisms, one possibility is that the circadian rhythm of melatonin, produced by the pineal gland, normally feeds back onto SCN cells to keep them synchronized (McArthur et al., 1991; Cassone et al., 1993; Reppert et al., 1994). Another possibility is that a diffusible factor produced by SCN cells may play a crucial synchronizing role. It has recently been shown, for instance, that agents liberating nitric oxide can shift the circadian phase of SCN neurons in a slice (Ding et al., 1994). Such a diffusible messenger may have been excessively diluted in the large extracellular volume of a culture dish. Finally, although interneuronal gap junctions have not been reported in rat SCN, it is possible that gap junctional communication among glia and/or neuron–glia interactions may be important for synchronization (van den Pol et al., 1992; Prosser et al., 1994). The MEP recording technique employed here is uniquely suited for testing such hypotheses.

Experimental Procedures

Cell Culture

SCN cells were obtained from 1- to 3-day-old Sprague-Dawley rats (Charles River) in a colony maintained on a light cycle of 12 hr light, 12 hr dark (lights on at 0700 hr). Dissections were performed at midday, during the light portion of the light–dark cycle. Cylindrical punches of unilateral SCN were made from 400 μm coronal sections, using a 20-gauge needle. Cells were dissociated using papain and cultured according to the procedure of Baughman et al. (1991). Neuronal cell density was about 3000 cells per square millimeter for MEP recordings, and 300 cells per square millimeter for other studies. Cells were grown in MEM (GIBCO 11430-014) supplemented with 3.6 g/l D-glucose, 26 mM $NaHCO_3$, 0.5 mM glutamine, 25 U/ml penicillin, 25 μg/ml streptomycin, and 5% rat serum. Medium was replaced once or twice per week. To control glial proliferation, cultures were treated 1–2 weeks after dissociation with 10 μM cytosine arabinoside (Sigma), a mitotic inhibitor.

Immunolabeling

Immunolabeling was performed by standard methods (Banker and Goslin, 1991), using fluorescent or biotinylated secondary antibodies (Vector Labs). Primary antibody sources: Z334, polyclonal to GFAP, DAKO; R10N, polyclonal to GABA–BSA, INCSTAR, gift of M. D. Johnson; VP–NP, polyclonal to VP neurophysin, gift of F. Grant and A. Robinson (Roberts et al., 1993); VIP-3, polyclonal to VIP, gift of P. Riskind (Riskind et al., 1989); SOMA-10, monoclonal to SS, gift of J. C. Brown (Vincent et al., 1985); αSV2, monoclonal to SV2, gift of K. Buckley (Buckley and Kelly, 1985); PS-45, monoclonal to neurophysin, gift of H. Gainer (Whitnall et al., 1985). Labeling was eliminated by omission of primary antibody or, for VP-NP, VIP-3, and SOMA-10, by preabsorption with 10 μg/ml immunogen.

Patch Recordings

Whole-cell patch recordings of spontaneous synaptic currents were performed using an EPC-7 amplifier (Adams and List), by standard methods (Hamill et al., 1981). During recording, cells were maintained in HEPES-buffered Ringer's solution (pH 7.4; 20°C–25°C), which contained 140 mM NaCl, 10 mM NaOH–HEPES, 4 mM KCl, 2 mM $MgCl_2$, 1 mM $CaCl_2$, and 20 mM glucose. Standard intracellular solution (pH 7.2) contained 119 mM KCl, 10 mM KOH–HEPES, 5 mM EGTA, and 2 mM $MgCl_2$. Some experiments used a more physiological internal solution (pH 7.3) containing 135 mM K methane sulfonate, 15 mM creatine phosphate (Tris), 10 mM HEPES, 5 mM KCl, 5 mM MgATP, 1 mM GTP (Tris), and 0.5 mM EGTA (Na). Both internal solutions contained 1% Neurobiotin (Vector Labs). CAP recordings of spontaneous action potentials (Forda et al., 1982; Johansson and Arhem, 1992) were performed under the same conditions as for whole-cell recordings, except that pipettes contained Ringer's solution. Both types of patch recordings were conducted primarily at hours coincident with the light portion of the rats' light–dark cycle.

Multielectrode Plate Recordings

MEPs for extracellular recording of action potentials from cultured neurons were originally developed by Gross et al. (1977) and by Pine (1980). We used MEPs containing a hexagonal array of 61 microelectrodes (Pine and Gilbert, 1982, Soc. Neurosci., abstract; Regehr et al., 1989), supplied by Jerry Pine. Electrodes were circles of electroplated platinum 10–15 μm in diameter. Adjacent electrodes were 70 μm apart, and the entire array was 0.5 mm across. Electrodes were connected to the edge of the plate by leads of indium tin oxide, which were insulated by an overlying layer of polyimide. A surrounding dish held about 1 ml of medium and formed a small culture well 6 mm in diameter. Except for the platinum electrodes themselves, MEPs were virtually transparent, allowing visualization of cultures. For recording, a MEP was fit into a recess in a circuit board containing multiplexors and preamplifiers (Regehr et al., 1989). The entire circuit board assembly was maintained at 37°C inside a tissue culture incubator containing an atmosphere of 5% CO_2, nonhumidified to avoid electrical artifacts. The culture dish interior was kept humid by a loosely fitting cover with moistened filter paper adherent to its underside.

When electrode impedances were low (100–500 kΩ at 1 kHz) and cell density was high (>1000 cells per square millimeter), spikes were detected from about 50% of cultures, with an average of 12 electrodes active per culture. Extracellular voltage signals from up to 8 electrodes at a time were amplified 10,000 × and filtered to a bandwidth of 3 kHz. Amplified signals were digitized into the memory of a Macintosh IIci computer at 10.4 kHz per channel (MacADIOS II, G. W. Instruments, Somerville, MA). Amplitude, width, and time of voltage pulses exceeding preset recording thresholds were detected on-line by custom software. Off-line analysis eliminated low amplitude noise and assigned clusters of spikes of similar amplitude and width to individual cells (Meister et al., 1994). Cluster definitions were adjusted every few days to accommodate slow changes in spike amplitude. Presence of a clear refractory period was used as a criterion to assure that each spike cluster reflected activity of a single neuron (see Figure 3). No cell was detected on more than 1 electrode, but sometimes 2 or 3 cells could be recorded from a single electrode.

Statistical Analysis

Quantitative CAP data on neuronal firing at different times were compared by ANOVA or t tests (two-tailed). Categorical data were compared by χ^2 analysis. Statistically significant circadian rhythmicity was defined by the existence of a single, statistically significant ($p < .01$, one-tailed) periodogram peak in the range 20–28 hr, using the χ^2 periodogram (Sokolove and Bushell, 1978; Refinetti, 1993). For recordings at 4 hr intervals, circadian rhythmicity was evaluated by computing the best least-squares fit of the data to a 24 hr cosine (Bloomfield, 1976). For circadian phase determinations, firing rate data were smoothed using a 2 hr moving average. Circadian phase differences between cells were then calculated as the time in hours between the smoothed peaks of their circadian firing rhythms.

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