intensity at the cell body of the SCN neuron) was calculated in order to monitor $[Ca^{2+}]_c$ variations.

Measurement of Intracellular Ca^{2+} Using Yellow Cameleon

Yellow cameleon genes (YC3.6 [25]) linked to a neuron-specific enolase promoter (pNSE/YC) or to a cytomegalovirus promoter (pCMV/YC) were transfected into rat or mouse SCN cultures using a Helios Gene Gun system (Bio-Rad Laboratories, Hercules, CA, USA), as described in earlier work [16,17]. The technique involves coating small gold particles (0.6 μm, 5 mg) with vector-carrying YC genes (20 μg) according to the manufacturer's instructions and then blasting them into the slice cultures at days 7 or 8 *in vitro*.

We used two different imaging setups for the cameleon imaging. The primary setup was designed for Ca^{2+} imaging analysis at a high sampling rate. In this setup, images were acquired by an iXon EMCCD camera (Andor, Belfast, Northern Ireland, UK) mounted on an inverted microscope (IX71, Olympus) that was equipped with a 10× objective lens (LUCPlanFL, NA0.30, Olympus), a mercury arc lamp, and a Dual-view optical module (Optical-insights, Tucson, AZ, USA). For excitation we used a filter set (OI-05-Ex, Chroma Technology, Rockingham, VT, USA) that included an

excitation bandpass filter (436 ± 20 nm) and a dichroic mirror (455DCLP, Chroma). In order to minimize the photo-bleaching, two neutral-density filters (ND6, ND50, Olympus) were placed in the excitation light path as well. The fluorescent emission was separated by a dichroic mirror (505DCXR, Chroma) and two emission bandpass filters (480 ± 30 nm and 535 ± 40 nm) installed in the Dual-view module. An electromagnetic shutter (Vincent Associates, Rochester, NY, USA) was placed in front of the lamp house. The shutter control, image acquisition, and online image processing were coordinated by software based on the Andor SDK subroutine packages. A SCN slice that had successful YC expression was transferred to the recording chamber and superfused with warmed oxygenated regular ACSF. The fluorescent emission images were collected every 1.5 seconds for more than 5 minutes. The acquired images were further analyzed by ImageJ (NIH, Bethesda, Maryland, USA) with custom-built software.

The second imaging setup was based on the original cameleon imaging system created for the long-term monitoring of $[Ca^{2+}]_c$ levels in SCN neurons [16,17]. That setup uses an inverted fluorescent microscope (Axiovert 405M, Carl Zeiss) equipped with a custom-built microscope stage CO_2 incubator, a mercury arc lamp, an excitation filter (435.8 nm DF10, Omega Optical, Brattleboro, VT, USA), an excitation neutral density filter (ND.5, Omega Optical), a dichroic mirror (455DRLP, Omega Optical),

and a 20× objective lens (Plan-Neofluar 20×, NA0.5, Carl Zeiss). The two emission bandpass filters (480DF30 and 535DF25, Omega Optical) are switched by a filter changer wheel (C4312, Hamamatsu Photonics, Hamamatsu, Japan). We acquired the resulting image pairs through a cooled CCD camera (C6790, Hamamatsu) at a sampling rate of 1 frame per 3 seconds for detecting Ca^{2+} spikes or of 1 frame per 10 minutes for detecting circadian $[Ca^{2+}]_c$ oscillations. Argus-HiSCA imaging software (Hamamatsu) was used to control an electromagnetic shutter (Copal, Tokyo, Japan), a filter changer wheel, and image acquisition.

Statistical Analyses

Means were calculated along with standard errors. Single pairwise comparisons were analyzed with the two-tailed Student's t-test. A one-way analysis of variance (ANOVA) was used to compare the population percentage of differently prepared SCN slices and the different Ca^{2+} spiking responses to different levels of fluo-4 AM concentration.

Results

Spontaneous $[Ca^{2+}]_c$ Spiking Activities in Fluo-4 AM Loaded SCN Slices

We prepared and stained the acute slices of SCN with a bath application of 20 μM fluo-4 AM (Fig. 1A and B) as described in Materials and Methods, and used a laser-scanning confocal microscope to monitor their short-term $[Ca^{2+}]_c$ activities over a duration of 800 seconds. Time traces exhibiting Ca^{2+} spikes are shown in Fig. 1C and D. Typically, the traces showed neither a discernable temporal pattern nor cell-specific characteristics: the width of each spike and the inter-spike interval both varied significantly in time and from one cell to the other (Fig. 1C and D). The observation time was limited due to dye bleaching. Therefore, we monitored a total of 2639 stained cells in 82 slices during various circadian phases and found that 18 ± 1% (mean ± SEM) of them exhibited spontaneous Ca^{2+} spikes. Collectively, our extensive set of data showed no indication of circadian variation. In other words, unlike the AP firing frequency of SCN neurons, the Ca^{2+} spiking frequency showed no circadian variation (Fig. 1E).

Right after the $[Ca^{2+}]_c$ imaging experiments were done, some slices were double-stained with an astrocyte maker, sulforhodamine 101 to determine if the active

cells producing Ca^{2+} spikes were a neuron or glia [23, 26]. The majority of the active cells turned out to be neurons (76 ± 5%, 81 out of 106 cells in 9 slices).

Absence of Sspontaneous Ca^{2+} Spikes in Cameleon-Transfected SCN Slices

We also looked for the occurrence of spontaneous Ca^{2+} spikes in rat and mouse slice cultures transfected with pCMV/YC or pNSE/YC. In the rat slice cultures, Ca^{2+} spikes were observed only in 2.8 ± 1.2% of the cells (number of slices = 5) transfected with pCMV/YC and in 0.6 ± 0.6% of the cells (number of slices = 5) transfected with pNSE/YC. In the mouse slice cultures, Ca^{2+} spikes were observed only in 1.2 ± 0.7% of the cells (number of slices = 5) transfected with pCMV/YC and were not observed at all in cells transfected with pNSE/YC (number of slices = 5). These percentages were much smaller than those for the acute rat SCN slices that were stained with fluo-4 AM ($F_{4,97}$ = 8.5, $P < .01$ by one way ANOVA; Fig. 2A).

Using mouse slice cultures transfected with pNSE/YC, we confirmed the presence of circadian rhythms in $[Ca^{2+}]_c$ in 62.8 ± 3.8% of the SCN neuronal population (27 out of 44 neurons; number of slices = 6; Fig. 2B). After monitoring the circadian $[Ca^{2+}]_c$ rhythms for several cycles, we imaged the SCN neurons at a much higher sampling rate (1 frame per every 3 seconds) for 5 minutes during different circadian

phases (Fig. 2C and D). There was no Ca^{2+} spiking activity even at the plateau phase.

Inducible Ca^{2+} Spikes in Cameleon-Expressed SCN Cells

To understand the lack of Ca^{2+} spikes in cameleon-transfected SCN cells, we examined the effect of additional fluo-4 AM loading on the cameleon-transfected SCN cells (Fig. 3A and B). At a concentration of 2.5 μM, fluo-4 AM evoked Ca^{2+} spikes (Fig. 3C) in 14% of the SCN cells examined (17 out of 118 cameleon-expressed cells in 4 slices). Similarly, the treatment with BAPTA-AM (2.5 μM) could also induce Ca^{2+} spikes in 13% of the SCN cells (20 out of 155 cells in 5 slices) (Fig. 3D). Since both were dissolved in DMSO, we also performed several DMSO control experiments to find no Ca^{2+} spikes (9 slices).

The Modulations in the Level of $[Ca^{2+}]_c$ and the AP Firing Pattern of SCN Neurons by Fluo-4 Loading

We made fluo-4 imaging and current clamp recording simultaneously using fluo-4 dye loaded micro-pipettes in order to see the direct effect of fluo-4 calcium dye on the level of $[Ca^{2+}]_c$ and the AP firing pattern in individual SCN neurons. Figure 4A shows a typical pattern of spontaneous AP firing in a SCN neuron in the absence of

fluo-4 dye. It is quite regular and shows a stable resting membrane potential (*Vm*). In many cases, this regular pattern changed significantly with an addition of fluo-4 (pipette concentration in the range of 80–120 μM). As the dye diffused from the patch pipette into the target neuron, during the first 100 seconds or so, the AP firing rate gradually decreased from 7 Hz to 3Hz, and at around 150 seconds the AP activity disappeared rather quickly (Fig. 4B). This change was accompanied by a decrease in *Vm* from -45.0 ± 1.6 mV to -54.0 ± 4.0 mV (Fig. 4C). Subsequently, the AP firing rate showed a large change, producing a random sequence of sharp peaks with each peak matched with a Ca^{2+} spike in one-to-one manner (Fig. 4B). During each peak, the value of *Vm* recovered more or less to the initial level (Fig. 4C).

Regarding the Ca^{2+} spikes in the SCN cells induced by fluo-4 dye, the following facts were further confirmed. First, the results were concentration-dependent. When the pipette dye concentration was below 60 μM, no Ca^{2+} spikes were apparent, and there was no significant change in either the AP firing rate or *Vm* (10 neurons in 6 slices; Table 1). Conversely, when the dye concentration was more than 150 μM, *Vm* and $[Ca^{2+}]_c$ increased steadily until the SCN neurons died (8 neurons in 4 slices). When the dye concentration was in the intermediate range of 80–120 μM, about half (9 out of 18 cells in 8 different slices) of the tested SCN neurons exhibited Ca^{2+} spikes, and they

showed a decrease in *Vm* of 6.7% ($P < .05$ by Student's *t*-test).

Fluo-4 AM Concentration Dependence of Ca^{2+} Spikes

The frequency of induced Ca^{2+} spikes was not a simple function of fluo-4 AM concentration. We observed quite heterogeneous responses following a stepwise increase of fluo-4 AM concentration (0.5-10 μM; Fig. 5). Based on these responses, SCN cell populations could be divided broadly into three different groups. For the majority of the cells (48.7%, 37 out of 76 cells in 3 slices), the amplitude as well as the frequency of Ca^{2+} spikes were an increasing function of fluo-4 AM concentration (Fig. 5A and B; Frequency: $F_{5,105}= 14.6$, Amplitude: $F_{5,105} = 3.8$, $P < .05$). For the next majority of the cells (35.5%, 27 out of 76 cells in 3 slices), there was no systematic trend (Fig. 5C and D) both in the frequency and the amplitude. Finally, for the rest of the cell population (15.8%, 12 out of 76 cells in 3 slices), the frequency was a decreasing function of fluo-4 AM concentration (Fig. 5E and F; $F_{5,33} = 7.74$, $P < .05$), while the amplitude could be decreasing, unchanging, or increasing, depending on the individual cells. In other words, the systematic trend in the frequency did not guarantee the same trend in the amplitude.

Discussion

The Rate of "Spontaneous Ca^{2+} Spikes" Showed No Circadian Variation

The spontaneous AP firing rates and the cytosolic concentration of free calcium ions $[Ca^{2+}]_c$ of SCN neurons are known to have circadian rhythms. Also reported was the existence of $[Ca^{2+}]_c$ transients in SCN having a typical duration of tens of seconds. Here, we termed these Ca^{2+} transients as "Ca^{2+} spikes." One immediate question following these observations would be if the Ca^{2+} spiking activity of SCN neurons can exhibit any circadian variation similar to the circadian AP firing activity, and here we studied that issue. The level of $[Ca^{2+}]_c$ was visualized by Ca^{2+} sensitive fluorescent protein cameleon as well as fluo-4 AM dye. Many fluo-4-loaded SCN cells exhibited Ca^{2+} spikes spontaneously, but our extensive set of fluo-4 imaging experiments based on 82 acute SCN slices found no day/night variation in the Ca^{2+} spiking rates (Fig. 1E): the Ca^{2+} spiking activities of SCN cells during the night time were as strong (or weak) as those of day time, or vice versa. In our earlier studies that investigated the progressive changes in $[Ca^{2+}]_c$ for the duration longer than a circadian cycle, we used organotypic cultures of SCN slices that were transfected by pNSE/YC genes [14,16,17]. The level of $[Ca^{2+}]_c$ was estimated with YC2.1 at every 10 minute in order to minimize photo-

bleaching: with this slow image acquisition rate, the presence or the absence of Ca^{2+} spikes could not be determined [16]. In the present study, we used YC3.6 (either pNSE/YC or pCMV/YC), and $[Ca^{2+}]_c$ images were acquired at every 3 seconds to monitor Ca^{2+} spiking events. The cameleon YC3.6 is a newer version of YC2.1 giving a better spatiotemporal resolution [25]. We observed that YC3.6-transfected organotypic cultures of SCN could exhibit well-defined circadian rhythms in the level of $[Ca^{2+}]_c$, just as in the YC2.1-transfected cultures. However, in the same preparations almost no Ca^{2+} spike was observed even at the peak of $[Ca^{2+}]_c$ circadian oscillation (Fig. 2). This was definitely not due to the lack of sensitivity of YC3.6 because spontaneous Ca^{2+} spikes were observed in other hyperthalamic neurons neighboring SCN (Figure S1).

"Spontaneous Ca^{2+} Spikes" Were Induced by Fluo-4 Ca^{2+} Dye Loading

Our study found Ca^{2+} spikes to be present in about 18% of fluo-4-loaded SCN cells, while Ca^{2+} spikes were observed only in 1-3% of cultured SCN cells, which expressed pCMV/YC genes, and in 0-0.6% of cultured SCN neurons, which expressed pNSE/YC genes. In addition, the population of cultured SCN cells that displayed Ca^{2+} spikes was significantly increased to about 15% by an additional loading of fluo-4 AM or BAPTA-AM (Fig. 3). Taken all together, we could conclude that the observed

spontaneous SCN Ca^{2+} spikes were not intrinsic to SCN but induced by fluo-4 dye loading.

In order to verify the effect of calcium dye loading on the level of $[Ca^{2+}]_c$ and the spontaneous AP firing activity in SCN neuron, more directly, we performed a patch-clamp recording along with fluo-4 imaging. Most notably, the loading of fluo-4 through the patching micro-pipette to the cell initially decreased both the AP firing rate and the level of resting membrane potential (Fig. 4) of SCN neurons. The decreases were not due to the whole-cell patching itself, as no such decrease was observed in our control experiments. Soon after the spontaneous AP firing activity of SCN neurons disappeared completely, an intermittent sequence of AP firings in groups, each of which well matched to a Ca^{2+} spike in one-to-one manner, emerged (Fig. 4B). The bursting mode of AP firings is quite unusual for SCN neurons, since their spontaneous AP firing pattern is typically regular as shown in Fig. 4A.

It has been proposed that Ca^{2+} influx via L-type Ca^{2+} channels [9] or BK type calcium-activated potassium channel activities [27] act to regulate spontaneous AP firing frequencies in SCN neurons. On the other hand, Aguilar-Roblero et al. [28] have demonstrated that AP firing frequencies in SCN neurons can be increased by ryanodine receptor activators (e.g., caffeine) and decreased by ryanodine receptor blockers (e.g.,

dantrolene): in other words, the mobilization of Ca^{2+} store by ryanodine-sensitive intracellular Ca^{2+} channels can modulate AP firing rate. In any case, all of these studies indicate a close connection between the level of $[Ca^{2+}]_c$ and the AP firing activity of SCN neurons, and thus it is not too surprising that in our whole-cell patching experiment each Ca^{2+} spike matches with a group of bursting AP spikes in one-to-one manner.

Although our current investigation did not answer how exactly the chelation of Ca^{2+} by dye loading interfered with the cell's intrinsic calcium kinetics to produce Ca^{2+} spikes, there is no doubt that they were induced by fluo-4 dye loading. The observed Ca^{2+} spikes could be a natural response of SCN neurons trying to recover their original cytosolic $[Ca^{2+}]_c$ levels. Some mechanisms were discussed earlier for the cause of SCN Ca^{2+} spikes. For example, van den Pol et al. [18] and Obrietan and van den Pol [19] found that they could be induced by the modulation of neurotransmitter, such as gamma-aminobutyric acid (GABA) and glutamate. Also, more recently, Irwin and Allen [20] demonstrated that the Ca^{2+} influx through L-type Ca^{2+} channels following the electrical stimulation of retinohypothalamic tracts could induce Ca^{2+} spikes in SCN neurons. The Ca^{2+} spikes observed in our experiments were neither induced by neurotransmitter modulations nor electrical stimulations. Yet, they were visually very

similar to the ones reported earlier [18-20]. In any case, the SCN Ca^{2+} spikes all were not an intrinsic spontaneous activity but induced.

Differences between Cameleon and Fluo-4 Calcium Dye

It has been known that BAPTA-based high affinity calcium dyes, such as fura-2, fluo-3, and fluo-4 can have a significant intracellular Ca^{2+} buffering and induce some non-intrinsic phenomena. For example, Ca^{2+} waves supported by the network of astrocytes can be completely blocked by the chelating action of fura-2 [29]. They can also delay the calcium signals initiated by extracellular calcium influx or release of calcium from intracellular stores by 5- to 20-fold [30]. Therefore, it is not too surprising that Ca^{2+} spikes in SCN neurons have appeared as an artifact of the Ca^{2+} chelation of fluo-4 dye.

When the chelation by indicator dye becomes an issue, its affinity (the inverse of dissociation constant K_d) and concentration are two important factors to be considered. High-affinity chelators trap calcium efficiently, and the presence of them at high concentration can sequester intracellular free Ca^{2+} significantly. We believe that the affinity (K_d = 345 nM) and the concentration (5 ~ 20 μM) of fluo-4 were high enough to interfere with the intrinsic calcium kinetics of SCN neurons and resulted in non-intrinsic

Ca^{2+} spikes. The affinity of cameleon YC3.6 (K_d= 250 nM) is 1.73 times higher than that of fluo-4 (K_d = 345 nM). Therefore, we suspect that the cytosolic concentrations of cameleon protein expressed within the SCN neurons were quite lower than that of fluo-4 dye that we used. As for the concentration, another important factor to be considered is the stability of the dye. It is well known that cameleon is far more stable than any BAPTA-based calcium indicators with respect to dye leakage, exhaustion, or cumulative photodestruction [31]. Therefore, the effective concentration of cameleon inside the cells could have been much lower than that of the fluo-4 dye.

The concerns with BAPTA based dyes are well known and our extensive set of data provides a clear case for concern, especially, regarding SCN calcium dynamics. However, our result does not invalidate the use of these dyes.

SCN Cells Are Heterogeneous with Regard to Their Cytosolic Ca^{2+} Buffering Systems

Previous research has shown that Ca^{2+} buffering molecules, such as calbindin D-28k (K_d is about 300 nM), may play a critical role for the function of SCN neurons [32,33]. The type of Ca^{2+} buffering proteins and their localization within the SCN may differ depending on the particular animal species [34,35], the developmental stages [36],

and the circadian timings [37]. For example, Ikeda et al. (2003a) has shown that the nucleus and the cytoplasm of SCN neurons can support quite different Ca^{2+} kinetics at a given time.

Several earlier studies found BAPTA-based fluorescent Ca^{2+} dyes often compete with the Ca^{2+} buffering system of involved cells. In fact, the Ca^{2+} chelating effects of fura-2 could be used to estimate the capacity of the Ca^{2+} buffering systems in various types of cells [38-40]. Accordingly, we suspect that the heterogeneous effect of fluo-4 AM on the generation of Ca^{2+} spikes (Fig. 5 and Table 1) may reflect the different Ca^{2+} buffering capacities of SCN cells. The SCN neurons have been categorized into several different subgroups [41,42], and it is quite possible that the heterogeneity of the Ca^{2+} buffering systems may underlie the existence of these subgroups.

Concluding Remarks

Our experimental studies demonstrated that Ca^{2+} spikes, having a typical bandwidth of tens of seconds, could be induced in SCN neurons by the Ca^{2+} chelating effect of BAPTA-based dyes. The firing rate of these induced SCN Ca^{2+} spikes did not show a circadian variation. Furthermore, Ca^{2+} spikes were very rarely observed when a reporter protein was used instead. Based on these facts, we conclude that spontaneous

Ca^{2+} spikes in the presence of BAPTA-based dyes are most likely not related to the intrinsic circadian rhythm of $[Ca^{2+}]_c$ in SCN neurons. At this point, however, we need to indicate several other possibilities as well. First of all, our study does not exclude the potential importance of Ca^{2+} spikes, for example, which are related to the modulation of neurotransmitters, for various functions of SCN. Second, it is also possible that there could have been a small subset of cells whose Ca^{2+} spiking rate indeed showed a circadian variation but masked by other non-circadian cells. We also realize that some cortical neurons can support very small and fast Ca^{2+} transients having a bandwidth of about a second or less. Such very fast Ca^{2+} transients could have existed in our experiments but unnoticed due to the limitation of our image acquisition rate (~1 second). On the other hand, the possible existence of such neurons in SCN has never been addressed nor been documented in the past.

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