Hierarchical organization of the circadian timing system

Steensel, M.J. van

Chapter 4

Sleep states alter activity of suprachiasmatic nucleus neurons

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SUMMARY

The timing of sleep and wakefulness in mammals is governed by a sleep homeostatic process and by the circadian clock of the suprachiasmatic nucleus (SCN), which has a molecular basis for rhythm generation. By combining SCN electrical activity recordings with electroencephalogram (EEG) recordings in the same animal (the Wistar rat), we discovered that changes in vigilance states are paralleled by strong changes in SCN electrophysiological activity. During rapid eye movement (REM) sleep, neuronal activity in the SCN was elevated, and during non-REM (NREM) sleep, it was lowered. We also carried out selective sleep deprivation experiments to confirm that changes in SCN electrical activity are caused by changes in vigilance state. Our results indicate that the 24-hour pattern in electrical activity that is controlled by the molecular machinery of the SCN is substantially modified by afferent information from the central nervous system.

INTRODUCTION

Sleep is a regulated state of the brain that recurs every day and is hard to postpone. Its ultimate function is still unclear, but sleep has been implicated in many fundamental processes, such as memory function, brain energy metabolism and genetic programming[1–5]. According to a well-established model of sleep regulation[6], the timing of sleep and wakefulness is regulated by two independent processes: a sleep homeostatic process that increases during waking and decreases during sleep, and a circadian process that provides the sleep homeostat with a circadian framework.

During normal undisturbed sleep, the status of the sleep homeostat is reflected by the amount of slow-wave activity (SWA; characterized by EEG power density in the 1.0–4.0 Hz range) during NREM sleep, which is an indicator of the discharge of sleep need[6]. This has become evident from a clear dose–response relation between SWA and prior waking duration in many species of mammals, including humans[7–12].

The circadian regulation of sleep is less well understood, and electrophysiological studies in this direction are absent. Circadian processes are regulated by a 'pacemaker' that resides within the suprachiasmatic nuclei (SCN)[13]. This pacemaker is autonomous and functions as an adaptive system preparing the animal for behavior (waking, eating, sleeping) at the proper phase of the light/dark cycle. The circadian clock has a molecular basis for generating electrical activity rhythms[14]. Attributes of the electrical discharge rhythm depend on the presence of clock genes[15–18], whereas electrical activity itself is the major output of the circadian clock[19]. Recordings of SCN electrical activity *in vivo* in freely moving animals, kept in constant conditions, show that electrical activity is high during the subjective day, the part of the animals' rhythm that normally falls in the light, and low during the subjective night, the part of the rhythm that normally falls in the dark[20,21].

It has long been assumed that the timing of sleep is regulated independently of the need for sleep[6,22], but more recent data indicate that there is a continuous interaction between sleep homeostasis and the circadian clock[23,24].To investigate whether information on vigilance state reaches the circadian system, we analyzed the activity of the SCN in relation to the state of vigilance of the organism. This required the combination of long-term recordings of SCN neuronal activity in un-anesthetized animals and simultaneous EEG and electromyogram (EMG) recordings. To test for a causal relation between sleep states and SCN neuronal activity, we used two different types of sleep deprivation: a slow-wave deprivation during NREM sleep and, on a separated day, a total REM sleep deprivation. Both have been shown to be powerful tools to test sleep regulatory mechanisms[25–30]. Our data show that SCN neuronal activity is strongly and differentially affected by alternations among sleep states. These sleep state–dependent changes are superimposed on the circadian modulation in SCN activity. The finding that SCN neuronal activity correlates highly with fluctuations in SWA suggests that the SCN receives information

about changes in sleep homeostasis throughout the course of each 24-h cycle.

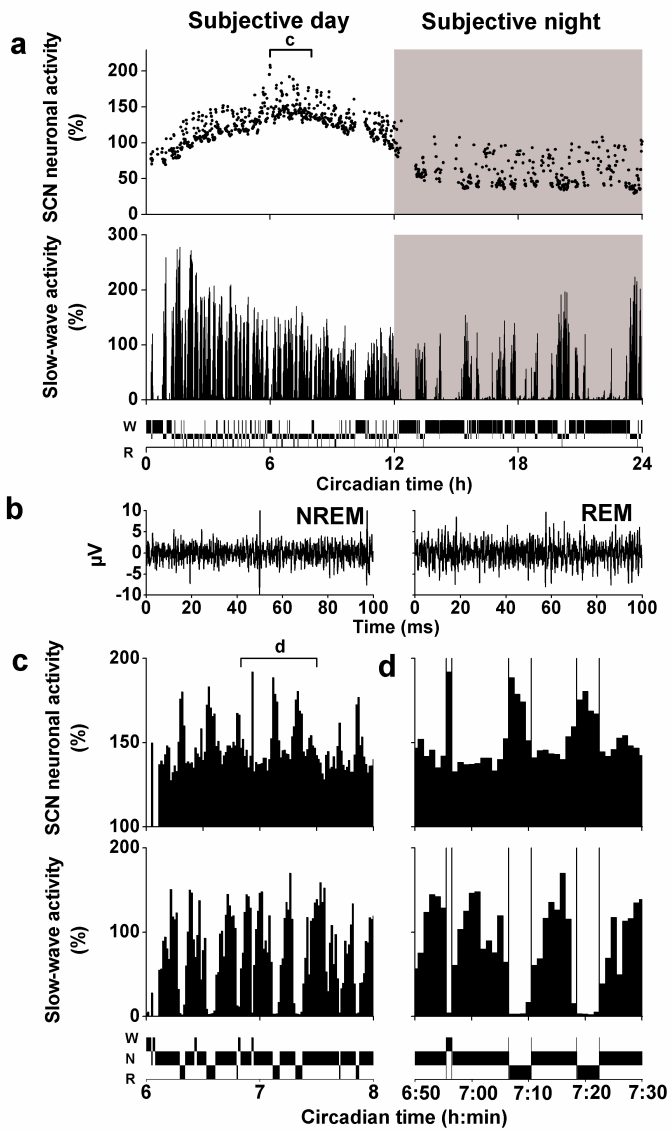

Figure 1. Vigilance states, slow-wave activity and SCN neuronal activity

(a) A 24-h record of SCN neuronal activity, SWA (EEG power density 1–4 Hz) and vigilance states (W, waking; N, non-REM sleep; R, REM sleep). Each data point is the mean of six 10-s epochs. SWA and SCN neuronal activity are plotted as a percentage of the mean activity during NREM sleep over 24 h. A gray background indicates the subjective night of the animal, with increased amounts of waking and lower SCN activity. (b) Two representative examples of oscilloscope traces with multiunit activity from both sleep states. Note the increased electrical activity during REM sleep. (c) Recordings of the same data as in a (top-left), zooming in on 2 h of the subjective day. (d) Recordings of the same data as in c (top-left), zooming in on 40 min. Vertical lines indicate vigilance state transitions. SWA shows high values during NREM sleep (N) and low values during REM sleep (R) and waking (W).

RESULTS

For each SCN recording, activity was higher during the subjective day than during the subjective night (Fig. 1a). Independent of the time of day, a clear relationship between vigilance states and SCN activity could be observed. Each time the animals entered REM sleep, neuronal activity in the SCN increased, and at the end of REM sleep, it decreased (Fig. 1b–d). Neuronal activity also increased during episodes of waking. These vigilance state–related changes were analyzed separately around the circadian peak (mid-subjective day) and the nadir (mid-subjective night) of SCN activity (Fig. 2). Our data show that SCN activity changes in parallel with changes in vigilance state, independent of circadian phase.

To investigate whether changes in SCN activity are related to changes in the activity of different frequencies in the EEG, we correlated EEG power densities of 0–25 Hz (grouped into 1-Hz frequency bins) with SCN neuronal activity for NREM sleep and REM sleep separately (Fig. 3a). In NREM sleep, significant correlations were exclusively obtained in the slow-wave range, with the highest negative correlation in the 2-Hz bin (r = –0.38, P < 0.0001). In contrast, during

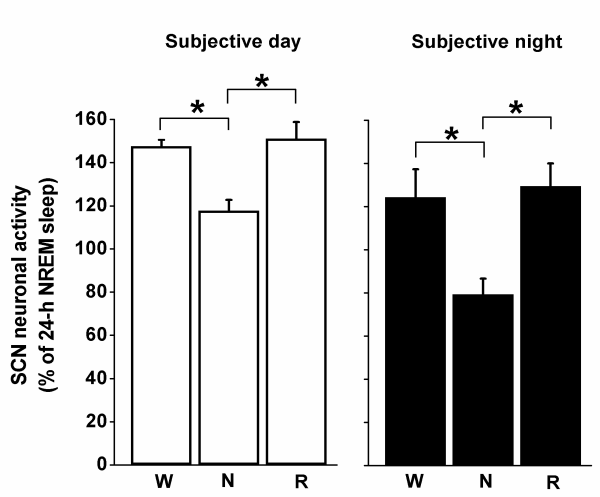

Figure 2. Suprachiasmatic nuclei neuronal activity

Mean SCN neuronal activity (± standard error of the mean (s.e.m.), n = 7) within the different vigilance states (W, waking; N, non-REM sleep; R, REM sleep) at the peak of activity during the subjective day (from 1.5 h before the peak to 1.5 h after the peak) and at the trough of neuronal activity during the subjective night (from 1.5 h before the trough to 1.5 h after the trough). SCN neuronal activity is plotted as a percentage of the mean activity during NREM sleep over 24 h. Asterisks indicate significant differences between NREM sleep and the other two vigilance states within the same circadian phase (P < 0.05, two-tailed paired t-test). SCN neuronal activity during NREM sleep differed significantly between subjective day and night (P < 0.05, two-tailed paired t-test).

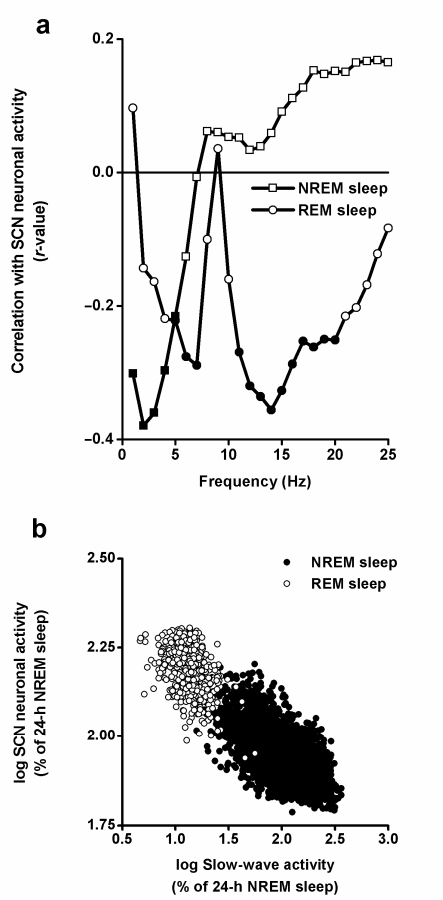

Figure 3. Correlation between SCN neuronal activity and EEG power density

(a) Mean *r*-values resulting from a linear correlation between the time course of the log-transformed EEG power density values in 1-Hz bins from 0 to 25 Hz and SCN neuronal activity during NREM or REM sleep. Values are means of individual *r*-values (n = 7) after Fisher-*z* transformation. Significant correlations are indicated by filled symbols ($P < 0.05$ after Bonferroni correction). Note that highest significant correlations are obtained in the slow-wave range (below 5 Hz) and within the frequency range of sleep spindles (11–14 Hz). (b) Example of individual data showing the relation between EEG power density in the slow-wave range (mean power density 1.0–4.0 Hz) and SCN neuronal activity for NREM sleep and REM sleep. Both variables are expressed as a percentage of their respective mean activity within NREM sleep over 24 h and are plotted in logarithmic values. In both vigilance states, the correlation between slow-wave activity and SCN neuronal activity was significant (NREM sleep: r = –0.612, $P < 0.0001$; REM sleep: r = –0.411, $P < 0.0001$).

REM sleep, significant correlations were obtained in the 6- and 7-Hz bins and in the sigma and low beta range (11–20 Hz), with the highest negative correlation in the 14-Hz bin (r = –0.36, $P < 0.0001$). These data indicate that the relation between SCN neuronal activity and EEG power density not only differs according to vigilance state, but also according to frequency bin within the same vigilance state. The strongest correlations were found within the slow-wave

range and the frequency range of sleep spindles. The correlation of SWA with SCN neuronal activity for one individual is shown in Fig. 3b; a smooth continuous transition from NREM sleep to REM sleep is visible. Also within NREM and REM sleep, a significant correlation was obtained between SCN neuronal activity and SWA in the EEG. At vigilance-state transitions, the changes in SCN neuronal activity were a mirror image of the changes seen in SWA (Fig. 4), which is in accordance with the significant negative correlation between the two variables during sleep.

To test the causal relationship between sleep state and SCN neuronal activity, we carried out selective sleep deprivations. EEG analysis revealed that the slow-wave deprivation during NREM sleep was successful in that SWA during NREM sleep remained below baseline levels (Fig. 5a). During slow-wave deprivation, neuronal activity in the SCN remained high and never decreased to the values it normally had during deep NREM sleep (Fig. 5a–c). This is confirmed by the finding that the mean level of SCN neuronal activity during NREM sleep was significantly higher during slow-wave deprivation than during NREM sleep in the control condition (Fig. 5d). REM sleep deprivation prevented significant increases in SCN neuronal activity (Fig. 5e–h). These results confirm that a causal relation exists between SCN neuronal activity and changes in sleep states.

DISCUSSION

Our data show that the activity of SCN neurons changes in parallel with the sleep/wake cycle and with the NREM/REM sleep cycle. SCN neurons show increased activity during REM sleep and waking. These vigilance state–related changes are superimposed on the circadian rhythm in SCN neuronal activity. The effects could be studied during both phases of the circadian cycle because the nocturnal rat does not show consolidated sleep and wakefulness. Notably, the vigilance state–related alterations in SCN electrical activity and the amplitude of the circadian cycle were of similar magnitude (Figs. 1,2,5).

The instantaneous changes in firing rate of SCN neurons showed high temporal correlation with SWA (1.0–4.0 Hz) and, to a lesser extent, with sigma (11.0–14.0 Hz) activity. EEG slow-waves and spindles are accurate indicators of sleep homeostasis and seem to be fundamental to the sleeping brain[31]. In humans, circadian variation of SWA during the initial part of sleep has been observed[32,33]. Here we show that SWA and SCN neuronal activity correlate significantly during NREM sleep.

A causal relation between vigilance states or SWA during NREM sleep and SCN neuronal activity was confirmed by the sleep deprivation experiments. When REM sleep or SWA was prohibited, SCN neurons did not show the observed increase or decrease in electrical activity, respectively (Fig. 5). Thus, changes in SCN electrical activity are caused by activation of sleep-associated input pathways, indicating that vigilance state has a strong influence on SCN neuronal activity. The present findings explain the absence of ultradian

fluctuations in electrical activity *in vitro*, when the SCN is de-afferented from most of the central nervous system[20]. The SCN receives cholinergic input from the nucleus basalis and from the pedunculopontine tegmental nucleus and the laterodorsal tegmental nucleus[34]. The latter two areas are known to be involved in REM sleep regulation[35]. In addition, the serotonergic projections from the raphe dorsalis[36], which are involved in NREM/REM sleep cycling[35], constitute a potential pathway for feedback of vigilance state onto the SCN.

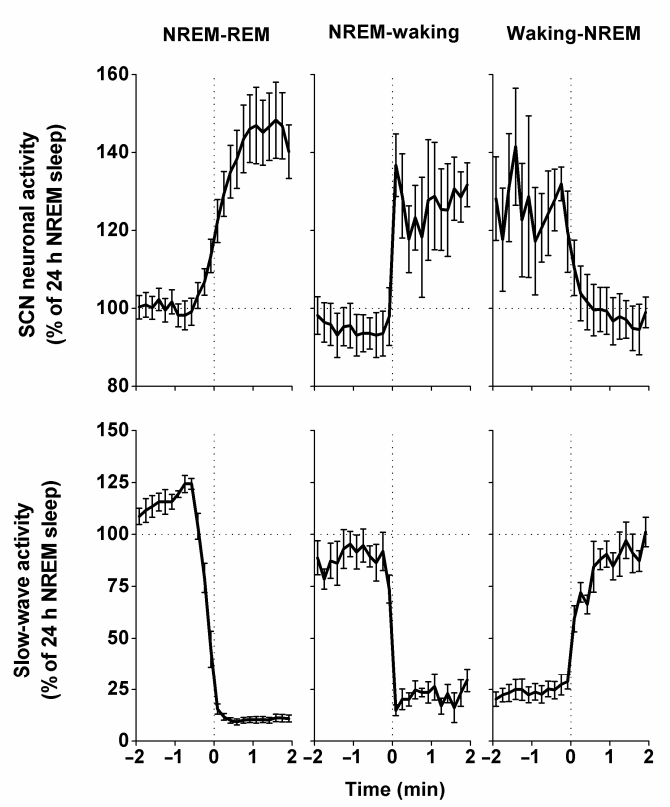

Figure 4. Vigilance state transitions

Time course of suprachiasmatic nucleus (SCN) neuronal activity (top panels) and EEG slow-wave activity (power density 1.0–4.0 Hz, bottom panels) at the transition from NREM to REM sleep, NREM sleep to waking, and waking to NREM sleep in the 2 min before and after the vigilance state transition. The curves connect 10-s mean values calculated over the entire circadian cycle. All variables are expressed as a percentage of the mean activity within NREM sleep over 24 h. All changes at the transition were significant ($P < 0.001$, ANOVA factor 'time' over 24 10-s epochs, $n = 7$).

It has generally been assumed that homeostatic and circadian sleep regulatory processes function independently[22]. Homeostatic responses in sleep persist after circadian rhythmicity has been abolished by SCN lesioning[37–39], and the circadian process can be manipulated by light in the morning without

changing SWA[40]. Our present results show that the circadian timing system is especially responsive to REM sleep and slow waves during NREM sleep. The amount of SWA in NREM sleep corresponds with the discharge of sleep need[6]. Thus, electrical activity in the SCN is determined not only by the molecular machinery of the circadian clock, but also by the amount of sleep need that is discharged during NREM sleep at a particular phase. During undisturbed sleep, discharge of sleep need is proportional to sleep pressure, indicating that circadian phase and the need for sleep can be integrated at this level.

Although it is clear that an animal's vigilance state affects SCN firing rate, it is unclear whether it also affects the transcriptional-translational loop of the molecular clock. Alternatively, the changes in firing rate could occur downstream from the clock at the level of the membrane potential. Sleep deprivation reportedly affects circadian phase[41] and clock gene expression[42], indicating that the first option is plausible. On the other hand, rigorous sleep deprivation may affect the SCN in a more severe way and cause effects that are unlikely to occur in an undisturbed situation. Apart from the issue of whether or not the molecular clock is affected, it is clear that the firing rate of the SCN, and thus the output of the circadian clock, is affected by vigilance state. This is of conceptual importance in showing that circadian patterns in electrical activity that are molecularly controlled can be influenced by the behavioral state of the animal.

METHODS

Animals

All experiments were performed under the approval of the Animal Experiments Ethical Committee of the Leiden University Medical Center. Subjects were seven male Wistar rats (300 g at the time of surgery).

SCN multi-unit recording

In vivo SCN recording techniques were as described previously[21]. Briefly, under deep anesthesia, animals were implanted with tripolar electrodes (stainless steel, diameter 0.125 mm; Plastic One). Two electrodes were aimed at the SCN with a distance of 0.4 mm between the electrodes. The third electrode was placed in the cortex for reference. Measurements were performed through one electrode at a time.

EEG and EMG recording

For EEG, electrodes were screwed through the skull on the dura over the right cortex (2.0 mm lateral to the midline, 3.5 mm posterior to bregma) and the cerebellum (at the midline, 1.5 mm posterior to lambda). For EMG recordings, two wires with suture patches were inserted between the skin and the neck muscle tissue. The animals were connected to the recording system by a

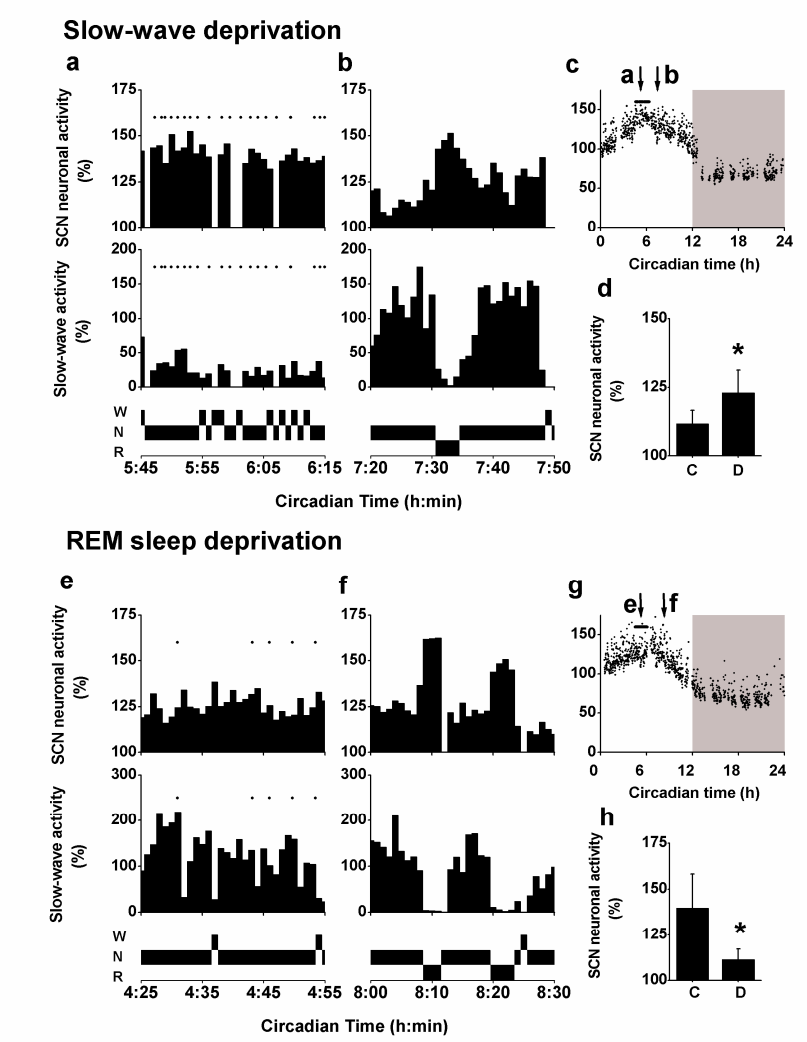

Figure 5. Deprivation studies

Effects of slow-wave deprivation during NREM sleep (a–d) and REM sleep deprivation (e–h) during the rest phase on vigilance states, SCN neuronal activity and SWA (EEG power density 1–4 Hz). EEG SWA and SCN neuronal activity are plotted as a percentage of their mean activity during NREM sleep over 24 h. (c,g) Gray backgrounds indicate subjective dark period; lines indicate time and duration of deprivation; arrows and letters indicate the area of zoom of panels a, b, e and f. Dots in a and e indicate time points of slow-wave and REM sleep deprivation, respectively. Note that during the slow-wave deprivation, SCN neuronal activity remained high (a), whereas when the animal was allowed to express SWA, SCN neuronal activity decreased as SWA increased (b). When REM sleep was prevented (e), the increase in SCN neuronal activity disappeared. The REM sleep–associated increase in neuronal activity returned as soon as REM sleep was allowed (f). (d,h) The difference in SCN neuronal activity under control conditions (C) and during slow-wave or REM sleep deprivation (D). Data for the slow-wave deprivation were collected during NREM sleep (d). For the REM sleep deprivation, control data were collected during REM sleep, and deprivation data were collected in the first minute after the intervention to prevent REM sleep (h). The astericks (d,h) indicate a significant difference between control and deprivation in both conditions (paired *t*-test, $P < 0.05$, $n = 3$).

flexible cable and a counterbalanced swivel system, and they remained on the cable in continuous darkness for at least one week before the start of the recording. The animals' drinking rhythm was recorded continuously to obtain an estimate of circadian phase.

Neuronal activity of the SCN was recorded on-line (amplified ~40,000×, band-pass filtered 500–5,000 Hz, –40 dB/decade) and processed further offline. On-line, a window discriminator converted the action potentials to electronic pulses. A second window discriminator was set at a higher level to detect artifacts caused by the animal's movements. SCN action potentials and movement artifacts were counted per 10-s epoch. The EEG and EMG were continuously recorded. The EEG and EMG signals were amplified (~2,000×), band-pass filtered (0.5–30 Hz, –40 dB/decade) and subjected to AD conversion (sampling rate 100 Hz). All data were recorded simultaneously and stored on a computer hard disk. The stability of the multi-unit signal and the EEG was evaluated on a daily basis by visual inspection of the signal on an oscilloscope and by monitoring the circadian rhythm in the signal and the amplitude of the EEG for an entire week before the baseline data were collected. After the experiments, we confirmed that the signals returned to baseline levels, and the animals were killed to verify the recording sites. To obtain a blue spot at the electrode tip, a current was passed through the electrode, and the brain was perfused with a solution containing potassium ferrocyanide. In all animals, a blue spot was obtained within the SCN.

Off-line EEG power density spectra were calculated in 10-s epochs corresponding to the 10-s epochs of the SCN action potentials with a fast Fourier transform (FFT) routine within the frequency range of 0.25–25.0 Hz in 0.1–Hz bins. EMG signals were integrated over 10-s epochs. Three vigilance states— waking, NREM sleep and REM sleep—were determined visually on the basis of standardized EEG/EMG criteria for rodents[43,44]. Waking was scored when the EMG showed an irregular pattern with high amplitude and the EEG showed low amplitude with relatively high activity in the theta band (6–9 Hz). NREM sleep was scored when EMG amplitude was low and the EEG amplitude was higher than waking, with high values in the slow-wave range (1–4 Hz). REM sleep was scored when the amplitude of the EMG and EEG were low and the EEG showed relatively high values in the theta range. Epochs containing artifacts in the SCN electrical signal or the EEG signal (observed during the scoring of the vigilance states) were excluded from analysis of the SCN neuronal activity and spectral analysis of the EEG, leaving approximately 5,098 10-s data points per animal per circadian cycle (41.4 ± 4.9% of recording time was excluded, with >80% of artifacts occurring during waking, $n = 7$).

All SCN neuronal activity data and EEG power density data were calculated relative to the mean value during NREM sleep over 24 h. This enabled us to calculate mean values over all animals. To analyze changes of EEG power density in SWA and changes in SCN neuronal activity at vigilance state transitions, intervals with a duration of 4 min containing artifact-free transitions from one

vigilance state (VS1) to another (VS2) were selected by the following criteria[44,45]. In the 2 min preceding the transition, at least 75% had to be scored as VS1, and not more than two epochs of VS2 were allowed. Similarly, in the 2 min after the transition, at least 75% had to be scored as VS2. Furthermore, the three 10-s epochs preceding and following the transition had to belong to the vigilance state corresponding to the transition.

Sleep deprivation

To test the causal relation between sleep states and SCN neuronal activity, we carried out two experiments using three animals. On two different circadian cycles, we applied either a slow-wave deprivation during NREM sleep, or a REM sleep deprivation. Both deprivations lasted for 2 h. The slow-wave deprivation was based on on-line EEG data. Whenever the animals were in NREM sleep for 20–30 s and high-amplitude slow waves were observed, we disturbed the animal by tapping on the cage, prohibiting the animals to enter deep NREM sleep[29,30]. Similarly, REM sleep was prohibited by stimulating the animals whenever an unambiguous REM sleep episode was recognized on the basis of the EEG[28,29].

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