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Photic and non-photic modulation of the mammalian circadian clock

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CHAPTER 2

Correlation with behavioral activity and rest implies circadian regulation by SCN neuronal activity levels

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ABSTRACT

The SCN of the hypothalamus contains a major pacemaker, which exhibits 24-h rhythms in electrical impulse frequency. Although it is known that SCN electrical activity is high during the day and low during the night, the precise relationship between electrical activity and behavioral rhythms is almost entirely unknown. The authors performed long-term recordings of SCN multiple unit activity with the aid of implanted microelectrodes in parallel with the drinking activity in freely moving mice. The animals were kept in a 12h:12h light-dark cycle (LD 12:12) and in short-day (LD 8:16) and long-day photoperiods (LD 16:8). Onsets and offsets of behavioral activity occurred when SCN discharge was around half-maximum value. Of the onsets 80%, and of the offsets 62%, occurred when SCN electrical activity differed less than 15% from the half-maximum electrical activity levels. Transitions between rest and activity could be described by a sigmoid shaped probability curve with Hill coefficients of 7.0 for onsets and 5.7 for offsets. The similarity in the onset and offset levels shows an absence of hysteresis in the control of behavioral activity by the SCN. Exposure to short- or long-day photoperiods induced significant alterations in the waveform of electrical activity but did not affect SCN electrical activity levels at which behavioral transitions occurred. In all photoperiods, the SCN signal was skewed with more rapid discharge changes during onsets (19% per hour) than offsets (11% per hour). The precision of the circadian system appears optimized, as transitions between behavioral activity and rest occur when the change in SCN electrical activity is maximal, both during the declining and rising phase. The authors conclude that transitions in behavioral state can be described by a probability function around half-maximum electrical activity levels and that the parameters of the SCN, predicting onset and offset of behavior, are remarkably insensitive to environmental conditions.

INTRODUCTION

Mammals display 24-h rhythms in behavior and physiology that are an adjustment to the 24-h period of the earth's rotation. The rhythms originate in a central circadian pacemaker located in the SCN of the hypothalamus and are synchronized to the environmental light-dark cycle via the retinohypothalamic tract (Weaver, 1998; Morin and Allen, 2006; Moore, 2007; Vansteensel et al., 2008). The generation of circadian rhythmicity in the SCN is explained by interactions among clock genes and their products, which lead to a cyclic protein expression with a period of approximately 24 h (Takahashi et al., 2008). The protein rhythms affect membrane properties, leading to rhythms in electrical activity that can be measured from SCN neurons (Welsh et al., 1995; Kuhlman and McMahon, 2006). The SCN control the timing of behavioral and physiological output parameters through electrical and humoral mechanisms that enable the animal to adapt to the environmental day (Schwartz et al., 1987; Silver et al., 1996; Kalsbeek et al., 2008).

SCN electrical activity can be recorded in freely moving animals using implanted microelectrodes. It has been shown that the electrical activity displays a 24-h rhythm, with high levels during the day and low levels during the night in both nocturnal and diurnal species (Inouye and Kawamura, 1979; Sato and Kawamura, 1984; Meijer et al., 1998; Yamazaki et al., 1998; Nakamura et al., 2008). In nocturnal rodents, the electrical activity of the SCN is thus inversely related to their behavioral activity pattern.

While alterations in the behavioral activity pattern are associated with changes in the electrical activity pattern within the SCN (Mason, 1991; Zlomanczuk et al., 1991; Mrugala et al., 2000), the precise relationship between SCN firing rate levels and the timing of behavioral activity remains relatively unexplored. Several formal models have accounted for the regulation of the activity time (α) and rest phase (ρ ; Wever, 1962; Aschoff et al., 1971; Enright, 1975; Pittendrigh and Daan, 1976; Davis and Menaker, 1980), but no evaluation of the activity profile of the central pacemaker was performed, simultaneously with a behavioral analysis of the circadian activity pattern. In our present studies we aimed to elucidate whether specific levels in SCN electrical activity are associated with the onset or cessation of behavioral activity and whether changes in α and ρ correspond to changes in these levels as was suggested by Wever (1960) and Aschoff et al. (1971).

We performed simultaneous recordings of SCN *in vivo* electrical activity and drinking activity and quantified the relationship between the firing rate level and behavioral onset and offset. Recordings were obtained from animals exposed to a 12:12h light-dark (LD) cycle and to constant darkness (DD). Additional studies in animals kept in long and short photoperiods were performed to determine whether day length affects the electrical activity threshold corresponding to onsets and offsets of behavioral activity. We also analyzed whether the characteristic acute increase in the level of activity during behavioral onset, versus the gradual decline of activity during the behavioral offset, reflects an asymmetry in SCN electrical activity patterns.

MATERIALS AND METHODS

Animals

Male adult C57BL6 mice (Harlan, Horst, the Netherlands) were individually housed under a lightdark cycle of 12 h of light and 12 h of darkness. Another group of mice was entrained to either a long (16-h light, 8-h dark) or a short (8-h light, 16-h dark) photoperiod for at least 30 days. Parts of the recordings from short and long photoperiods were previously used in vanderLeest et al. (2007). All experiments were performed under Institutional Animal Studies Committee approval.

Electrode Implantation

Before surgery, animals were fully anesthetized using a mixture of midazolam, fentanyl, and fluanisone. Tripolar stainless steel electrodes (125 μm , Plastics One, Roanoke, VA) were implanted in the brain under a 5-degree angle in the coronal plane using a stereotactical device. Two of these electrodes were polyimide insulated and twisted together with only the tips exposed. They were aimed at the SCN, 0.46 mm posterior to bregma, 0.14 mm lateral to the midline, and 5.2 mm ventral to the surface of the cortex (Paxinos and Franklin, 2001). The third electrode was placed in the cortex for reference. At the end of the experiment, animals were killed and the electrode placement was determined by histology. Recordings from animals in which the recording location was outside the SCN were excluded from the analysis.

Multiple Unit and Behavioral Activity Recordings

Following a recovery period of 7 days, the animals were placed in a recording chamber (surface = 40 \times 40 cm) with free access to food and water and a built-in drinking sensor. The implanted electrode was connected to a data-acquisition system

using a counterbalanced, low-torque electrical swivel to minimize strain on the electrode. Electrical activity was amplified and spikes were counted using amplitude-based spike triggers. Drinking behavior was used as a measure of behavioral activity. The number of spikes and the occurrence of drinking activity was stored in 10-sec bins. After several days of recording in light-dark conditions, the animals were released into constant darkness to record behavior and multiunit activity (MUA) in the absence of any masking influences of light.

Data Analysis

Electrical activity data were smoothed in Matlab (MathWorks, Inc., Natick, MA) using a penalized least-squares algorithm (see Schaap et al., 2001, and Eilers, 2003, for a detailed description of the algorithm). Importantly, this procedure does not introduce phase shifts and has no boundary effects (like Fourier filters). The smoothing parameter (λ) was adjusted to the lowest value yielding a single maximum per circadian cycle and fell between 10^9 and 2×10^{11} . The smoothed data were normalized in Igor Pro (version 6; Wavemetrics, Lake Oswego, OR) by setting the trough to 0% and the peak to 100% for each cycle (**Fig. 1**). Onsets and offsets of drinking activity were determined by visual inspection of the actogram by 3 investigators independently, without knowledge of the corresponding MUA traces. Only onsets and offsets on which all 3 agreed were included in the analysis. Drinking onsets and offsets were scored over several days ($n = 10$ -16 days) and used to determine the electrical activity levels at the time points of the behavioral transitions using the normalized electrical activity pattern. The rate of change of SCN electrical activity was calculated from the first derivative of the normalized electrical activity. Statistical analyses were carried out with SPSS (version 16.0; SPSS Inc., Chicago, IL). A 2-way ANOVA with post hoc paired-sample t tests was used to compare MUA levels and rates of change in MUA at behavioral onsets and offsets. Differences between individual animals in the timing of behavioral transitions and half-maximum SCN firing rate levels relative to the light-dark cycle were analyzed with a 1-way ANOVA. To test for possible differences in the MUA level or in the rate of change in MUA during behavioral onsets or offsets between the different photoperiods we used a 3-way ANOVA. The width of electrical activity peaks in short- and long-day photoperiods were determined at half-maximum firing rate level and were compared with the time difference between behavioral offsets and onsets, which corresponds with the duration of the resting period, also defined as ρ (Aschoff, 1960). Differences in results between short and long days were evaluated with a t test. The number of animals (5 in each photoperiod) was insufficient to determine regional differences in electrical activity patterns within the SCN.

To investigate whether behavioral onsets and offsets occur when the rate of change of SCN firing rate is maximal, the average time difference between behavioral transitions and the peak rate of change were calculated per animal. A 1-sample *t* test was used to test the null hypothesis that the time difference equals zero. To test whether the variance of the time difference between behavioral transitions and the SCN electrical activity parameters is different between behavioral onsets and offsets we used the Morgan-Pitman test for correlated variances. In all tests, data were checked for normal distribution using a Kolmogorov-Smirnov test, and $p < 0.05$ was considered to be statistically significant. Data are given \pm SEM values.

RESULTS

Transitions between Rest and Activity around Half-Maximum SCN Firing Levels

Simultaneous recordings of SCN electrical activity and drinking activity in 5 animals kept in LD 12:12 and in constant darkness show high SCN firing rates and low behavioral activity levels during the light period or subjective day (**Fig. 1**). Average electrical activity levels in LD 12:12 were 49.4% ($n = 5$, ± 3.6) of maximal firing rate during onsets of drinking behavior and 51.8% (± 3.1) during behavioral offsets (**Fig. 2A**). In constant darkness, average electrical activity levels were 53.1% ($n = 5$, ± 1.3) during behavioral onsets and 55.6% (± 3.8) during behavioral offsets. Electrical activity levels did not differ significantly between behavioral onsets and offsets nor between LD and DD circumstances (2-way ANOVA, $p = 0.55$). Variability that was present in the timing of onsets and offset around the 50% MUA level (**Fig. 2C**) could be fitted by a Gaussian curve (**Fig. 2B**).

Inter-individual Differences in Behavioral Offset Timing

The electrical activity patterns of individual animals under LD 12:12 were compared with the behavioral drinking profiles (**Fig. 2D**). The time of behavioral onset (range: ZT 11.6 to 12.0) and the time of half-maximum firing rate (range: ZT 11.6 to 12.3) on the declining slope of the electrical activity rhythm did not differ significantly between animals ($n = 5$, 1-way ANOVA, $p = 0.052$ and 0.168 , respectively). However, significant variation between animals was present in the time of behavioral offset (ZT 23.1 to 0.3, $p < 0.001$) as well as in the time of half-maximum firing rate on the rising slope of the electrical activity rhythm (ZT 22.7 to 1.0, $p < 0.01$).

Photoperiod Affects the SCN Waveform but not the 50% Level Separating Behavioral Activity (α) and Rest (ρ)

Under the short photoperiod (Fig. 3A, LD 8:16, $n = 5$), electrical activity levels in LD were 53.8% (± 6.2) of maximal firing rate during the onset of drinking behavior and 45.4% (± 3.0) during behavioral offsets. During the first 4 days in DD, average electrical activity levels were 57.8% (± 8.1) during behavioral onsets and 48.4% (± 5.3) during behavioral offsets. Under the long photoperiod (Fig. 3B, LD 16:8, $n = 5$), average electrical activity levels in LD were 54.0% (± 3.4) during the onset of drinking

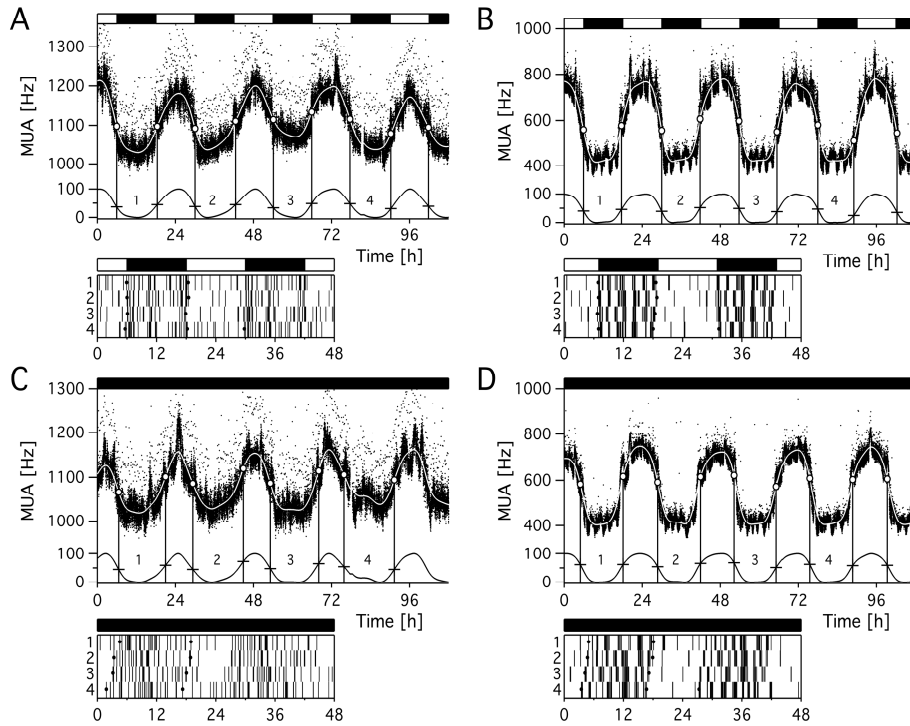


Figure 1. In vivo multiple unit activity (MUA) recordings and drinking actograms. (A-D) Graphs show representative SCN MUA recorded for approximately 4 days. A and B display data from animals recorded in a 12:12 light-dark cycle. C and D show recordings from the same animals in constant darkness. Black dots represent the number of spikes recorded in 10-sec bins. MUA was smoothed (white line) and normalized between 0% and 100% (black line, depicted below the raw data). White dots and vertical lines indicate the times when onsets and offsets of drinking activity occurred. Double plotted actograms (small graphs) show drinking activity of the mice during the recorded period. Onsets and offsets of drinking activity are indicated by black dots in the actogram. The number of the day is indicated both in the actogram and in the MUA recordings.

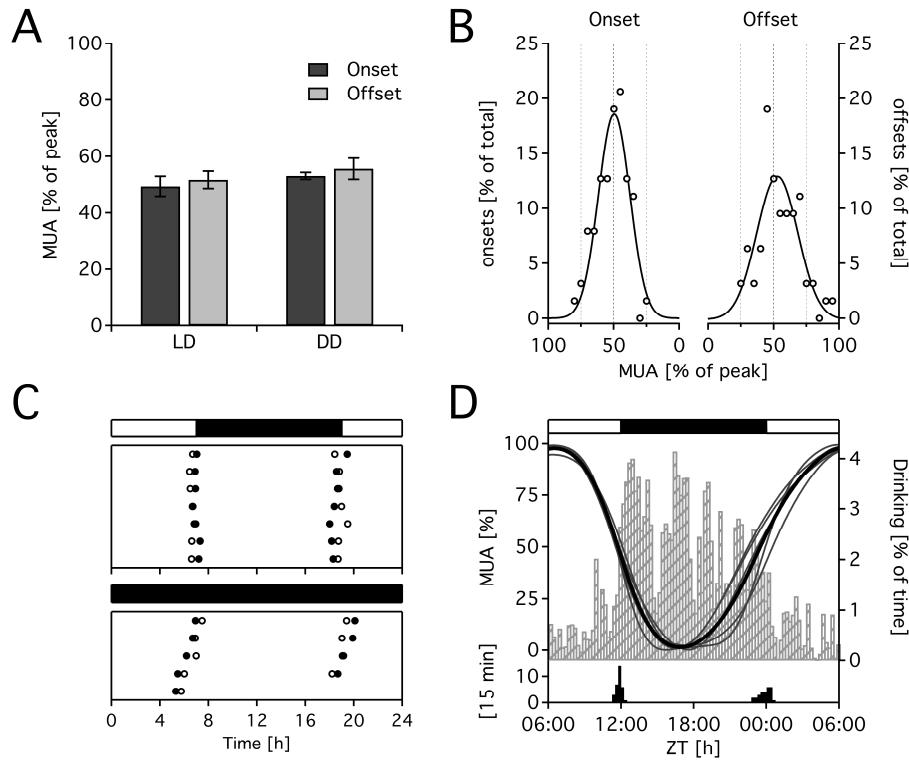


Figure 2. Multiunit activity (MUA) levels corresponding to onset and offset of behavioral activity. (A) Mean (\pm SEM, $n = 5$) percentage of electrical activity at which onsets and offsets of drinking activity occurred in animals kept in a 12:12 LD cycle (LD) or in constant darkness (DD). There are no significant differences between LD and DD or onset and offset (2-way ANOVA, $p = 0.55$). (B) Frequency distribution of the observed MUA levels during onsets ($n = 70$) and offsets ($n = 63$) of drinking activity. Individual onsets and offsets were counted in bins with a width of 5% of the MUA amplitude (open circles) and were fitted by a Gaussian distribution. Eighty percent of the drinking activity onsets and 62% of the offsets occurred when SCN electrical activity differed less than 15% from the half-maximum. (C) Half-maximum SCN electrical activity (open circles) and behavioral onsets and offsets of an individual animal (closed circles) plotted as an actogram. The upper actogram displays 7 consecutive days in LD 12:12 and the lower actogram shows 5 consecutive days in constant darkness. (D) The mean 24-h waveform of SCN electrical activity from 5 animals recorded in LD 12:12. The thick black line represents the average MUA waveform of all animals. Gray lines indicate the MUA waveforms of individual animals. The average drinking activity is depicted in 15-min bins (striped bars). The light-dark cycle is indicated above the plot. At the bottom, the timing and number of onsets and offsets of drinking activity is indicated in 15-min bins. Significant variation between animals was found in the time of behavioral offsets ($p < 0.001$, 1-way ANOVA) and in the time of half-maximum firing rate on the rising slope of MUA ($p < 0.01$, 1-way ANOVA), but not in the time of the declining slope.

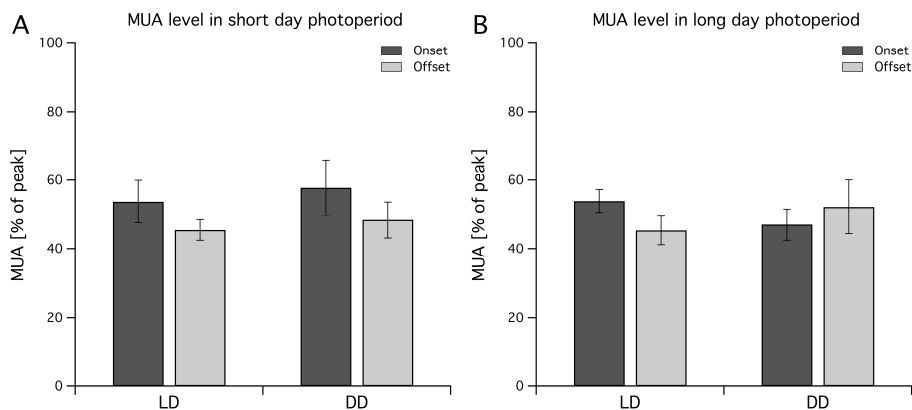


Figure 3. Multiunit activity (MUA) levels at behavioral activity onsets and offsets during photoperiodic entrainment. Animals were entrained to either a short (A; LD 8:16, $n = 5$) or long photoperiod (B; LD 16:8, $n = 5$). MUA and drinking were recorded in LD and constant darkness (DD). Data from the first 4 days in DD are included. Bars represent the percentage \pm SEM of maximal electrical activity at which onsets and offsets of behavioral activity occurred. No significant differences were found between LD and DD or onset and offset in either photoperiod (2-way ANOVA, $p > 0.4$).

behavior and 43.3% (± 4.8) during offsets. During the first 4 days in DD, average electrical activity levels were 47.0% (± 4.6) during behavioral onsets and 52.2% (± 7.8) during offsets. No significant differences in electrical activity levels during behavioral onsets or offsets were found between LD or DD conditions or between short and long photoperiod (3-way ANOVA, $p = 0.65$). Under the short photoperiod, the width of the electrical activity peak at half-maximum electrical activity was 10.5 h (± 0.3) in LD and 10.1 h (± 0.6) during the first 4 days in DD. Under the long photoperiod, electrical activity peaks were significantly broader in LD (15.8 h ± 0.6 , t test, $p < 0.001$), as well as during the first 4 days in DD (14.6 h ± 0.6 , t test, $p < 0.001$). During exposure to short and long photoperiods and during the first 4 days in constant darkness, the peak width in MUA was similar to the duration of the resting phase r (paired-samples t test, $p > 0.4$).

Asymmetry in SCN Electrical Activity Waveform

Both during exposure to different photoperiods and during the subsequent 4 days in DD, we consistently observed a higher rate of change in electrical activity during onsets compared to offsets of behavioral activity (Fig. 4; $n = 5$ in all groups). In LD,

the mean rate of change was 19% per hour during behavioral onsets and 11.0% per hour during behavioral offsets. The difference was significant under LD in all 3 photoperiods (**Fig. 4A**; $p < 0.01$). In DD, the difference was similar and significant in animals from LD 8:16 (**Fig. 4B**; $p = 0.039$) and LD 16:8 ($p = 0.007$), but did not reach significance in animals from LD 12:12 ($p = 0.072$).

Behavioral Transitions Occur when MUA Rate of Change Is Maximal

To determine whether behavioral onsets and offsets occurred when the rate of change in SCN electrical activity was maximal, the normalized electrical activity and its first derivative were plotted relative to the time of onsets and offsets of behavioral activity (**Fig. 5**). Each plot shows data from an individual animal, recorded over

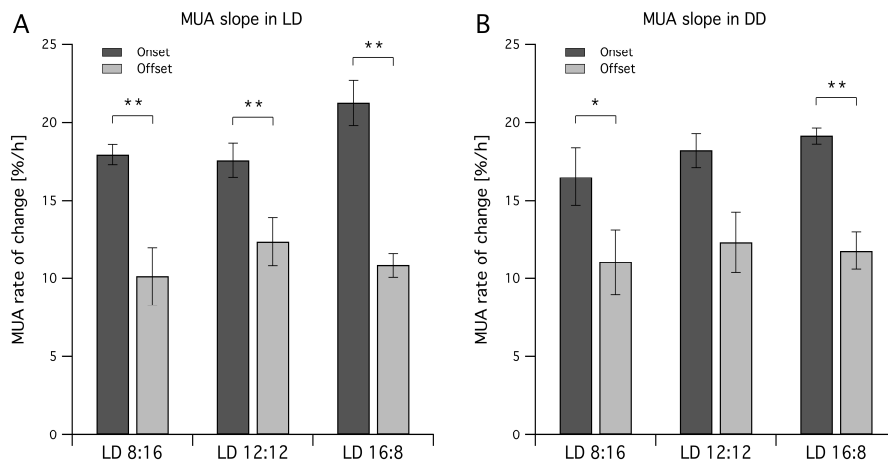


Figure 4. Slope of the electrical activity during the onset and offset of behavioral activity. Bars indicate the rate of change in electrical activity \pm SEM values, expressed as %/h of the amplitude of the rhythm. (A) Multiunit activity (MUA) rate of change during behavioral activity onset and offset for animals recorded in LD 8:16, LD 12:12, and LD 16:8. In all photoperiods, the rate of change in electrical activity is significantly larger during the onset of behavior compared to the offset. (B) MUA rate of change for animals recorded the first 4 days in DD after exposure to LD 8:16, LD 12:12, and LD 16:8. The rate of change in electrical activity is significantly larger during the onset of behavior compared to the offset after exposure to short- and long-day photoperiods (2-way ANOVA with post hoc paired t tests). After exposure to LD 12:12, the difference only showed a trend ($p = 0.072$). * $p < 0.05$, ** $p < 0.01$, n.s. = not significant; all tests are based on a 2-way ANOVA with post hoc paired t test.

several days in either LD 12:12 (Fig. 5A, C) or during subsequent DD (Fig. 5B, D). The behavioral transitions occurred when the rate of change in the SCN was maximal (1-sample t test, $p > 0.2$). The time difference between half-maximum SCN firing rate and behavioral transitions and the time difference between SCN maximum rate of change and behavioral transitions were both consistently smaller around behavioral onsets than around behavioral offsets. The phase relation between behavioral transitions and the time at which the rate of change in the SCN was maximal exhibited a standard deviation of 0.64 h for behavioral onsets in LD, 0.70 h for onsets in DD, versus 1.32 h for offsets in LD and 3.06 h for offsets in DD. Similarly, the phase relation between behavioral transitions and the half-maximum electrical activity exhibited an average standard deviation of 0.74 h for onsets in LD, 0.69 h for onsets in DD, versus 1.93 h for offsets in LD and 2.70 h for offsets in DD. When data from LD and DD were combined, a Pitman test showed a significant difference in variance between onsets and offsets ($p < 0.04$), but when separated the difference was below the significance threshold.

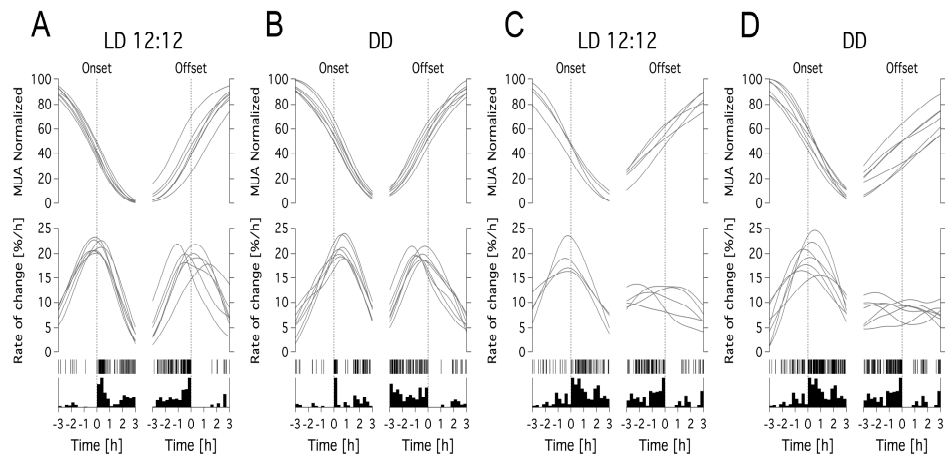


Figure 5. Temporal relationship between the onsets and offsets of behavioral activity and the rate of change of the electrical activity. Each panel represents data from a single animal recorded over multiple days in either LD 12:12 (panels A, C) or constant darkness (panels B, D). In each panel, the normalized electrical activity (top) and rate of change of the electrical activity are plotted in a 6-h window plotted relative to the time of drinking activity onset and offset ($T = 0$). Each gray colored line represents the electrical activity and corresponding rate of change on a single day. Vertical black lines show the occurrence of drinking activity in 10-sec bins. The bottom bar plot displays the drinking activity data displayed in 15-min bins.

Transitions in Behavioral State as a Probability Function

We plotted the observed onsets and offsets cumulatively as a function of the MUA level at which they occurred (Fig. 6). For activity onsets, we started at the peak of the electrical activity rhythm, while for activity offsets we started at the trough of the rhythm. Under all photoperiods, the likelihood of the occurrence of behavioral onsets and offsets as a function of SCN electrical activity could be described by a sigmoid curve. In LD 12:12, the fitted sigmoid function had a Hill coefficient of 7.0 ($R^2 = 0.99$) for the onsets and 5.7 ($R^2 = 0.99$) for the offsets. As the SCN discharge rate changes faster on the declining than on the rising slope, the time span in which the MUA level changes is shorter during behavioral onsets than during offsets. At the inflection point, the slope of the probability functions corresponds with a probability increase of 61% per hour for drinking activity onsets and of 30% per hour for the offsets.

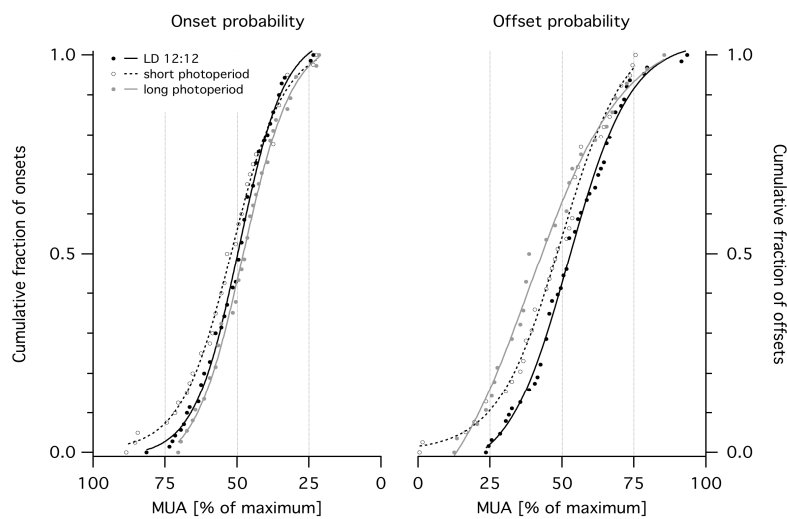


Figure 6. Behavioral onsets and offsets as a function of SCN electrical activity levels. Onsets and offsets of behavioral activity are plotted cumulatively as a function of SCN electrical activity levels. The horizontal axes show SCN multiple unit activity during the declining phase (on the left) and during the rising phase (on the right). Note that in the left panel, multiunit activity (MUA) levels on the x-axis start at 100%. Behavioral onsets (left) and offsets (right) are plotted separately for LD 12:12 ($n = 70$ onsets, 63 offsets) and short ($n = 40$ onsets, 39 offsets) and long photoperiods ($n = 37$ onsets, 28 offsets). A sigmoid function was fitted to the data. The % of MUA, depicted on the x-axis, corresponds with different timescales for onsets than for offsets, as the same trajectory (25-75%) is traversed in less time. Consequently, the chance for the occurrence of a behavioral onsets increases with a rate of 61% per hour around half-maximum electrical activity, versus a rate of 30% per hour for behavioral offsets.

DISCUSSION

Half-Maximum SCN Firing Rate Separates Behavioral Activity and Rest

For many physiological processes the response of the system to an input or driving force is not only dependent on the current state of the system, but also on its history. In this study we investigated the relation between electrical activity and transitions in behavioral activity, and questioned whether the relation shows path dependence or “hysteresis.” We show that transitions from behavioral activity to rest occur halfway on the rising slope of the electrical activity rhythm, while transitions from rest to behavioral activity occur halfway on the decreasing slope. The finding that onsets and offsets occur at the same SCN electrical activity level—irrespective of the direction of the change—indicates an absence of hysteresis in the regulation of behavioral activity by the SCN on a circadian timescale. A comparison of the SCN firing rate levels and behavioral activity in LD 12:12 revealed no differences in the time of behavioral onsets and half-maximum firing rate on the declining slope of SCN activity. On the other hand, inter-individual differences in the timing of behavioral offsets and half-maximum firing rate on the rising slope of SCN electrical activity were found. We conclude that inter-individual differences in α are mainly attributable to differences in the timing of the rising slope of SCN electrical activity, rather than to differences in the declining slope.

In our present studies, drinking activity was used to determine transitions in behavioral activity. Although previous studies have found a strong correlation between the circadian profiles of different behavioral parameters (Stephan and Zucker, 1972; Shibuya et al., 1980; Meijer et al., 1990), it may be questioned whether a different behavioral parameter (e.g., feeding or locomotor activity) would yield different onset and offset times. We added passive infrared motion detectors to record general locomotor activity in 2 experiments and found a 2.1% difference in the electrical activity level between onset of drinking activity and locomotor activity, which was not significant (paired-samples t test, $p = 0.09$). For the offset of locomotor activity, a 2.4% higher electrical activity level was obtained (paired-samples t test, $p < 0.001$). While this difference was present, it was small and falls within the observed range of electrical activity levels during drinking activity onsets and offsets. Importantly, the finding that the electrical activity levels do not differ significantly between onsets and offsets of drinking activity was also confirmed for locomotor activity (t test, $p > 0.05$).

While onsets and offsets of drinking activity occurred at half-maximum SCN electrical activity levels, deviations from the mean values were observed (**Fig. 2**). Although part of the variability may originate from some ambiguity in the estimation of onset and offset of activity, inspection of the individual actograms also revealed that very clear onsets and offsets corresponded with a range of firing rate levels, around the half-maximum. This suggests that the SCN control over the onset and cessation of behavioral activity does not operate on the basis of a single threshold value. Instead, the effects of SCN activity on behavioral transitions can be described by a probability function, acting around 50% SCN electrical activity levels, according to a sigmoid shaped curve (**Fig. 6**). We quantified the steepness of the slope of the sigmoid and observed a Hill coefficient of 7.0 for activity onsets and of 5.7 for activity offsets, indicating a tighter coupling between behavioral onsets and MUA than between offsets and MUA.

Effects of Photoperiod

Changes in photoperiod lead to adjustments in the width of the daytime peak in SCN electrical activity and changes in clock gene expression patterns, corresponding to changes in the duration of α and ρ (Mrugala et al., 2000; Nuesslein-Hildesheim et al., 2000; Sumova et al., 2003; Inagaki et al., 2007; Sumova et al., 2007; Tournier et al., 2007; VanderLeest et al., 2007; Yan and Silver, 2008; Naito et al., 2008; Brown and Piggins, 2009). We investigated whether modifications of the relationship between SCN firing rate levels and behavioral activity contribute to photoperiod-induced changes in α . Due to the sinusoidal shape of the SCN electrical activity waveform, increases in the threshold activity at which transitions in behavior occur would translate into a lengthening of α in nocturnal animals, while a decrease of the threshold activity would shorten α (Wever, 1960; Aschoff et al., 1971). Although such a mechanism would augment the photoperiodic encoding capacity of the circadian system, the data show no evidence of differences in the MUA level at which behavioral transitions occurred between the short and long photoperiod. Moreover, in both short and long photoperiods, we did not find a significant difference between the peak width at 50% of the MUA rhythm and ρ . Together, the data suggest that photoperiodic encoding relies on changes in the waveform produced by the SCN, but not by changes in the threshold for triggering activity and rest.

SCN Waveform Characteristics

The rate of change of electrical activity appeared maximal at the 50% level for all photoperiods, which contributes to the precision of the circadian timing system as large differences in electrical discharge levels occur within a limited time window

(Fig. 5). It implies that for the analysis of phase-shifting effects, the half-maximum firing rate seems not only mathematically more precise but also functionally more important than the commonly used peak time. The change in electrical activity was faster during behavioral onsets than during behavioral offsets in all photoperiods (Fig. 4). The consistent presence of this asymmetry indicates that it is a general feature of the mouse SCN firing rate pattern. Individual neurons of the SCN are active for relatively short periods of time and vary in the phase of the circadian cycle at which they display their maximum gene expression or firing rate levels (Schaap et al., 2003; Brown et al., 2006; Inagaki et al., 2007; vanderLeest et al., 2007; Naito et al., 2008). As the circadian waveform of the population is strongly dependent on the distribution of single unit patterns (Rohling et al., 2006), the skewness of the population pattern may reflect an asymmetrical distribution of SCN neurons.

Interestingly, the higher rate of change on the declining slope of the *in vivo* MUA waveform is not present when the SCN is recorded *in vitro* (vanderLeest et al., 2009). This suggests that the waveform is subject to modulatory influences of extra-SCN areas that are absent in an *in vitro* SCN preparation. One possible feedback mechanism could be formed by brain areas involved in behavioral activity. Inhibitory effects of behavioral activity on SCN firing were previously reported in hamsters and rats (Yamazaki et al., 1998; Schaap and Meijer, 2001). In this scenario, the increment in locomotor activity at the beginning of the subjective night may induce a feedback signaling to the SCN during the evening decline of firing rate. This process may play an active role in reinforcing behavioral activity onsets by accelerating the decline in SCN firing rate. In addition, the electrical activity of the SCN is modified by the arousal state of the animal. It has been shown that slow-wave activity (SWA) in the non-REM sleep EEG is negatively correlated with SCN firing rate (Deboer et al., 2003). Moreover, sleep depriving an animal during the first 6 h of the subjective day leads to an increment in sleep pressure and strongly reduces SCN electrical activity (Deboer et al., 2007). As sleep pressure and SWA reaches peak levels at the start of the rest phase, the inhibitory effect may dampen the slope of the morning rise of SCN electrical activity.

Regulation of Output Rhythms by the SCN

The SCN shows widespread neuro-anatomical projections to many parts of the central nervous system (Panda and Hogenesch, 2004; Kalsbeek et al., 2006) and interference with these projections abolishes circadian rhythmicity (Nunez and Stephan, 1977; Inouye and Kawamura, 1979; Honma et al., 1984; Eskes and Rusak, 1985; Abrahamson and Moore, 2006; Kalsbeek et al., 2008), suggesting that direct

neuronal output is important. On the other hand, behavioral rhythmicity is restored in SCN-lesioned animals receiving encapsulated SCN grafts, which allow for humoral but not neuronal output (Silver et al., 1996). Prokineticin 2 (PK2) and transforming growth factor- α (TGF- α) have been identified as a candidate SCN output molecule that regulates behavior (Kramer et al., 2001; Cheng et al., 2002; Zhou and Cheng, 2005). These findings have led to the unsolved question of whether humoral or neuronal output factors are essential for the ability of the SCN to control circadian rhythms in downstream structures. Our findings are not inconsistent with either of these views, since electrical activity within the SCN, as measured in the present studies, regulates both the electrical activity in output pathways and the release of humoral factors. The electrical activity profile within the SCN is therefore considered to be a first step in the formation of an output signal.

We do not infer from our data that the SCN drives behavioral activity at an ultradian timescale, nor that activity onset or offset reflect ultradian alterations (or variability) in the electrical discharge pattern. Therefore, we are not surprised that in a day-to-day analysis, half-maximum electrical activity levels do not fully correspond to behavioral onsets and offsets and that deviations from the 50% MUA level exist. However, our data do support a causal relation between electrical activity and behavioral activity on a circadian timescale. We consider the experiment in which we manipulated the length of the day a critical test to evaluate this relation. The manipulation of day length was expected to result in a change of the triggering levels for behavioral onset and offset (Wever, 1960, Aschoff et al., 1971). Our finding that the 50% level for triggering behavioral activity is preserved in short and long days, despite substantial waveform changes in the SCN, suggests a causal relation between SCN activity and behavior. Importantly, this experiment included an analysis of the first days in continuous darkness, excluding a possible immediate effect of light on SCN discharge levels.

In conclusion, our results indicate that within a certain range of electrical activity levels, transitions in behavioral state are most likely to occur (**Figs. 6, 7**). During the incremental phase of the electrical activity pattern, the driving force to arrest activity increases according to a sigmoid-shaped curve, while during the decremental phase the driving force to trigger activity increases according to a sigmoid. The best predictor for the occurrence of onsets and offsets is the 50% electrical activity level.

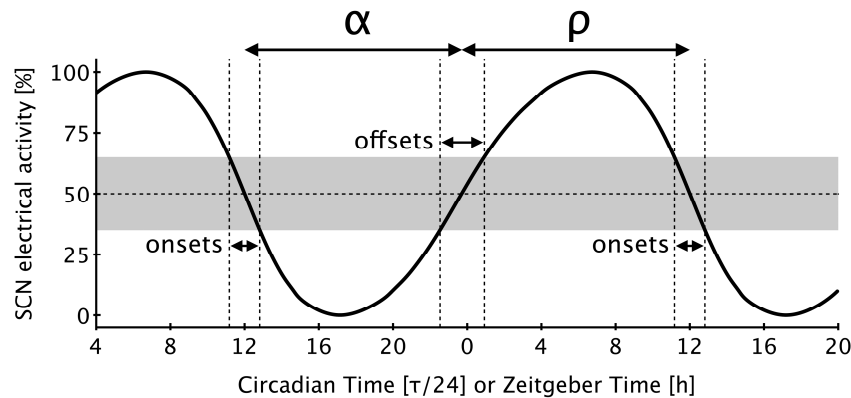


Figure 7. Relation between SCN electrical activity and the behavioral activity pattern. The observed relation between SCN firing rate and behavioral activity is summarized based on the average SCN waveform in LD 12:12 (black line). The onset and offset of behavior divide the circadian cycle into an active (a) and a resting phase (r). The half-maximum electrical activity (horizontal dashed line) is the best predictor for the occurrence of behavioral transitions. The variability in the relation between SCN activity level and behavior can be described by a sigmoid probability function with a working range centered around half-maximum multiunit activity (horizontal gray band). Under all LD cycles, the SCN electrical activity waveform was skewed, with a fast declining slope (mean rate of change during onsets: 19% per hour) and a more gradual inclining slope (11% per hour during offsets).

The precision of the system is optimized, as the rate of change, and thereby the change in the driving force, is maximal around the 50% values. While our findings agree in general terms with all formal models previously proposed (Enright, 1975; Pittendrigh and Daan, 1976; Davis and Menaker, 1980), the observed skewness of the electrical activity pattern and the precise regulation of activity onset versus activity offset are in close accordance with the kinetics predicted by Wever and Aschoff (Wever, 1962; Aschoff et al., 1971). While they treated the SCN pacemaker as a single oscillator, we now understand that a multitude of oscillators determines the waveform of the population signal. Unforeseen was the independence of the outcomes on photoperiod.

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