Photoperiodic encoding by the neuronal network of the suprachiasmatic nucleus

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CHAPTER 2

Phase of the Electrical Activity Rhythm in the SCN *in vitro* not Influenced by Preparation Time

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Summary

The mammalian circadian clock, located in the suprachiasmatic nucleus (SCN) of the hypothalamus, drives daily rhythms in behavioral, physiological, and endocrine functions. The SCN has a genetic basis for rhythm generation and remains rhythmic when it is isolated and kept in constant conditions. This allows for an *in vitro* analysis of circadian attributes, which is a powerful approach in the study of SCN cellular mechanisms. For studying the phase of the SCN rhythm *in vitro*, it is important to assess whether preparation of the tissue itself introduces phase shifts. In the present study, we investigated whether preparation of hypothalamic brain slices affects the phase and waveform of the rhythm in electrical impulse frequency of the mouse SCN. Mice were kept under a 12:12h light-dark cycle, and slices were prepared at six timepoints distributed over the 24h cycle. We used the peak time and the time of the half-maximum levels in electrical activity as markers for circadian phase. The peak time in electrical activity was observed during the mid-subjective day, irrespective of the time of preparation, at a mean ZT of 5.18 ± 0.20h (n = 39). After preparation in red light at the end of the subjective night, the circadian phase appeared slightly advanced. When slices were prepared in the dark, using infrared illumination, the ANOVA showed no significant differences in peak times and time of half-maximum values between preparation times. The results affirm the value of the slice preparation for studying the phase of the SCN *in vitro*. We conclude that the phase and waveform of the electrical activity in the SCN *in vitro* is unaffected by the time of slice preparation but may be influenced by short light presentation when preparation is performed during the subjective night.

Introduction

In mammals, circadian rhythms in physiological, endocrine, and behavioral functions are driven by the suprachiasmatic nucleus (SCN) (Stephan and Zucker, 1972; Meijer and Rietveld, 1989; Ralph et al., 1990). The SCN is located at the base of the anterior hypothalamus and receives light input via the retinohypothalamic tract (Morin and Allen, 2006). The ability of the SCN to generate circadian rhythms can be explained at the molecular level by a transcriptional-translational feedback loop (Takahashi, 1993; Kume et al., 1999; Reppert and Weaver, 2002; Dardente and Cermakian, 2007) Several genes have been identified that play an essential role in the generation of circadian rhythmicity, including Period (Per), Cryptochrome, Clock, and Bmal (Albrecht et al., 1997; Shearman et al., 1997; Hastings et al., 1999; van der Horst et al., 1999; Vitaterna et al., 1999; Vitaterna et al., 1994; Bunger et al., 2000; Hastings and Herzog, 2004). Electrical activity is a major signal of the SCN pacemaker (Schwartz et al., 1987) and shows clear circadian modulation in discharge rate, with high frequencies during the day and low frequencies during the night (Meijer et al., 1998). Neuronal and humoral pathways distribute this temporal information to other parts of the central nervous system (Abrahamson and Moore, 2001; Kalsbeek et al., 2006; Silver et al., 1996). Circadian rhythms in e.g. gene expression (Nakamura et al., 2005; Yamazaki et al., 2000), neurotransmitter content (Shinohara et al., 2000b), and electrical activity (Brown et al., 2006; Gillette et al., 1995; Schaap et al., 2003) persist in *in vitro* preparations, such as in dissociated cells (Welsh et al., 1995), in organotypic slice cultures (Herzog et al., 1997) and acutely prepared hypothalamic slices (Groos and Hendriks, 1982). The *in vitro* brain slice preparation offers the valuable possibility to investigate circadian rhythms under constant conditions and in the absence of influences from other parts of the central nervous system. Moreover in acutely prepared slices, it is possible to evaluate after-

effects of previous manipulations of the light-dark (LD) cycle, such as different photoperiods, constant light, or shifts of the environmental LD cycle (Albus et al., 2005; Jagota et al., 2000; Mrugala et al., 2000; Nagano et al., 2003; Nakamura et al., 2005; Ohta et al., 2005; Schaap et al., 2003; Shinohara et al., 1995; VanderLeest et al., 2007; Vansteensel et al., 2003b; Yannielli and Harrington, 2000).

For all such *in vitro* studies, it is important to determine whether the time of preparation of the slice affects the phase of the SCN rhythm, such as the time of maximal activity. However, detailed studies on this issue are scarce. One study reported that in Per1-luc transgenic rats, the time of preparation largely determines the peak time of the Per1 rhythm *in vitro* (Yoshikawa et al., 2005). These results prompted us to analyze potential phase-shifting effects of slice preparation on the electrical activity rhythm *in vitro* in more detail. We used three phase markers to evaluate the influence of slice preparation on the SCN rhythm: the time of maximum activity, the time of half-maximum discharge level on the rising slope, and the time of half-maximum activity on the declining slope. We also measured the broadness of the recorded electrical activity peaks. The results of the present study show that the time of preparation of brain slices has no influence on the different phase markers of the electrical activity rhythm in the SCN.

Methods

Animals

All experiments were performed under the approval of the Animal Experiments Committee of the Leiden University Medical Center and in accordance with the journals' ethical standards (Portaluppi et al., 2008). Male C57Bl6/J mice (Harlan, Horst, The Netherlands) were individually housed in clear plastic cages that were equipped with a running wheel, in a sound-attenuated and temperature-controlled room. Food and water were available ad libitum. The animals were entrained to a 12:12 LD cycle for at least two weeks, with the time of lights-off defined as Zeitgeber time 12 (ZT) and the time of lights-on as ZT 0. The animals were sacrificed at ZT 2, 6, 10, 14, 18, or 22. Additional experiments were performed at ZT 0, immediately after lights-on, because preparation at ZT 2 did not allow for a reliable estimation of the peak. Decapitation during the subjective day (at ZT 0, 2, 6, and 10) as well as subsequent brain dissection occurred under normal room light. Dim red light (10μW/cm^2) was used when the animals were taken from their dark period (ZT 14, 18, and 22). Additional experiments were performed at ZT 22 in the dark, with the aid of infrared light viewers.

In vitro Electrophysiology

Preparation of brain slices and recordings were performed as described earlier (VanderLeest et al., 2007). After decapitation, brains were rapidly dissected and placed in ice-cold bicarbonate buffered artificial cerebrospinal fluid (ACSF), containing (in mM): NaCl 116.4, KCl 5.4, NaH_2PO_4 1.0, $MgSO_4$ 0.8, $CaCl_2$ 1.8, $NaHCO_3$ 23.8, and glucose 15.1, as well as 5 mg/l gentamycine. Coronal, hypothalamic slices (~400μm) containing the SCN were prepared with a tissue chopper and transferred to a laminar flow chamber, where they were kept submerged in ACSF (35°C), and stabilized by a tungsten fork. The slice chamber was continuously perfused with ACSF at a rate of

1.5ml/min and oxygenated with warmed, humidified O_2 (95%) and CO_2 (5%). Before the recording was started, slices were equilibrated in the chamber for ~1h. One slice per animal was used and contained at least 50% of the rostro-caudal extend of the SCN. Recording electrodes (insulated 90% platinum and 10% iridium, 75μm) were placed in the ventro-medial aspect of the left and right SCN in order to obtain multiunit neuronal activity recordings from both nuclei. The electrical activity was amplified (×100k) by a high-impedance amplifier and subjected to a band-pass filter (0.3-3 kHz). A hardware spike trigger was set so that the threshold was above noise level. The action potentials crossing this threshold were counted in 10s bins with the aid of custom-made software. The electrodes and spike threshold settings were left unaltered during the experiment. Data were excluded from analysis if the fluid levels were unstable or when the phase markers could not unambiguously be determined.

Data Analysis

The data were analyzed offline in MATLAB (The Mathworks). The multiunit data were plotted against ZT and smoothed using a least squares algorithm (Eilers, 2003). Peak time, determined as the time of maximum firing, as well as minimum and half-maximum levels, were determined in these smoothed recordings. To estimate the influence of the smoothing parameter on the observed peak times, we applied both a weak and stronger smoothing procedure (weak: average $\lambda = 1 \times 10^{9}$; stronger: average $\lambda = 8 \times 10^{10}$). When both recordings of the slice were successful, we took the average from both channels, resulting in a more robust estimate of circadian phase of the whole slice (see statistical analysis); otherwise, we used the data of one recording. In all experiments, the first peak in multiunit activity was used as a primary marker of clock phase. Additionally, the times of half-maximum activity levels on the rising and declining slope of the multiunit activity patterns were calculated. We also calculated the width of the electrical activity peak, on the basis of the half-maximum values.

Statistical Analysis

Statistical analysis was performed in Origin7 (OriginLab Corporation, One Roundhouse Plaza Northampton, Massachusetts, USA). Before averaging the peak phases of two recordings from a single slice, we performed a two-sided, paired t-test on the peak phase between both recording channels from all successful experiments and found that peak times within a slice were not significantly different (paired t-test, $p > 0.06$). The influence of a stronger smoothing on the time of the maximum electrical activity was also tested using a two-sided, paired t-test. To test for differences among preparation times, one way ANOVAs with post-hoc Tukey and Bonferroni tests were used. We applied ANOVAs for time of maximum activity, half-maximum values and width of the peak. All provided values are means ± standard error of the mean (SEM). Data were considered significantly different when $p < 0.05$.

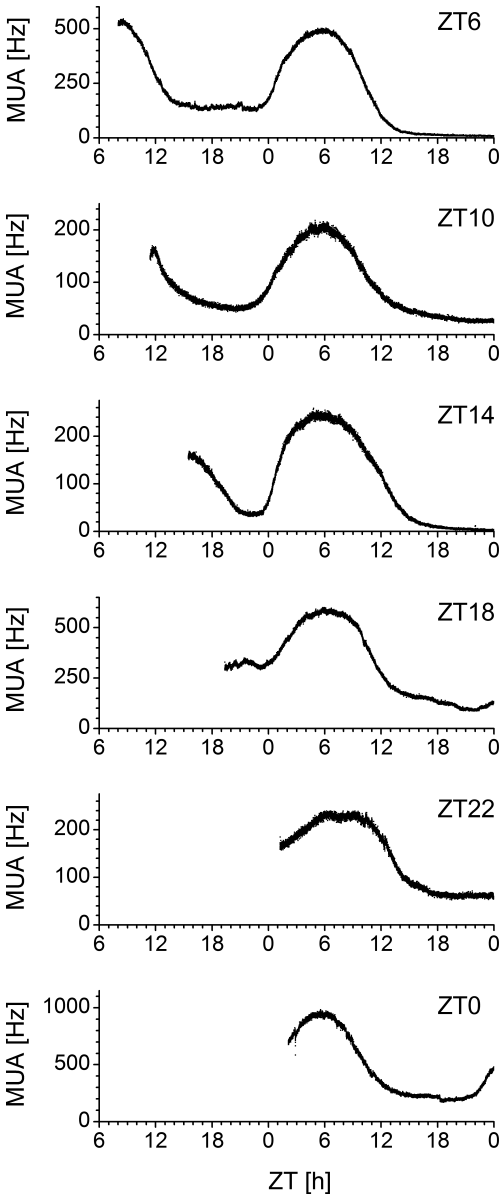


Figure 2.1

Extracellular multiunit electrical activity rhythms in the SCN following different preparation times.

Raw data plots of multiunit electrical activity as a function of Zeitgeber time. Preparation was started, from top to bottom, at ZT 6, ZT 10, ZT 14, ZT 18, ZT 22, and ZT 0, respectively. The timing of the peak and shape of the rhythm in spontaneous action potential firing rate is consistent between preparation times.

Results

We successfully recorded rhythms in spontaneous electrical activity in the SCN in a total of 51 acutely prepared hypothalamic slices. We obtained successful recordings from both SCN nuclei in 36 slices and from one nucleus in the other 15 slices. Our recordings following preparation at ZT 0, 6, 10, 14, 18, and 22 showed increased electrical activity during the projected day and decreased activity during the projected night (see Figure 2.1). All of our individual electrical activity peaks occurred during the projected light at a mean ZT of 5.18 ± 0.20h (n = 39; see Figure 2.2).

The mean peak times for preparations during the dark period of the animal were ZT 14: 5.76 ± 0.58h (n = 5), ZT 18: 5.43 ± 0.48h (n = 5), and ZT 22: 3.92 ± 0.53h (n = 6). The mean peak times obtained for preparations during the day were ZT 6: 4.36 ± 0.35h (n = 11) and for ZT 10: 5.97 ± 0.54h (n = 5). We performed additional experiments and prepared slices at ZT 0, because preparation at ZT 2 (n = 6) appeared too close before peak time and interfered with the estimation of the peak. The mean peak time after preparation at ZT 0 was 5.19 ± 0.25h (n = 6).

An ANOVA on the peak times obtained for the different times of preparation reached significance ($p < 0.02$), showing that the data were not homogeneous; however, neither a post-hoc Tukey nor a post-hoc Bonferroni test on the data used in the ANOVA revealed significant differences in peak time between any of the groups. Although not significantly different, the average peak time after preparation at ZT 22 appeared relatively early, with four of the ten experiments that displayed a peak before ZT 4 following preparation at ZT 22. Therefore, we performed additional experiments and prepared slices at ZT 22 in the dark with the aid of an infrared light viewer. This resulted in a mean peak time of 5.30 ± 0.62h (n = 7), which was not different from the results obtained in red light at ZT 22 (t-test, $p > 0.12$). Moreover, when we used these results in the

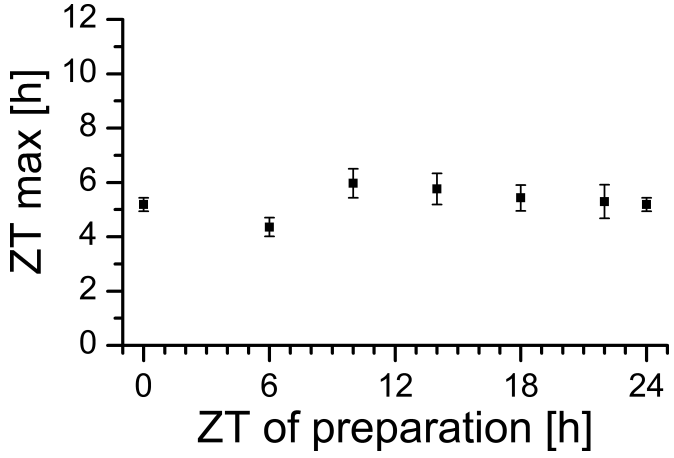


Figure 2.2

Peak phase in multiunit electrical activity following different preparation times.

The time of maximal MUA, or multiunit electrical activity (ZTmax ± SEM), is plotted against the time of brain slice preparation. All peaks in MUA occurred during the projected light period. No significant differences were observed in peak time when preparation in the subjective night was performed in the dark using infrared viewers (ANOVA, p > 0.15). Note that the ZT 0 and ZT 24 data points are repeated and thus are the same values.

ANOVA, no significant effect of the time of preparation was observed ($p > 0.15$).

Furthermore, we determined the peak times of all successful recordings after applying a higher smoothing parameter (Supplemental Figure 2.1). The stronger smoothing did not result in a consistent earlier or later peak time, but the SEM in peak time decreased slightly (weak smoothing: 5.17 ± 0.19h; stronger smoothing: 5.08 ± 0.17h; paired t-test, $p > 0.22$, n = 51).

During the rising and declining phase, the electrical activity changed rapidly (21.5 ± 1.3%/h and 16.9 ± 1.0%/h respectively, n = 24), offering a precise marker for circadian phase. We analyzed the times of the half-maximum levels at the rising and declining phase of the electrical activity rhythm; in this analysis, we used data from ZT 22 that were obtained in darkness (see Figure 2.3). The mean half-maximum level of the rising phase occurred at ZT 0.96 ± 0.17h (n = 24), and at the declining phase it occurred at ZT 10.28 ± 0.22h (n = 38). The mean peak width was 9.24 ± 0.29h (n = 24). No significant effects of preparation time were observed at any of the circadian

phases on the half-maximum values or peak width (ANOVA, rising slope: $p > 0.07$, n = 24; falling slope: $p > 0.06$, n = 38; peak width: $p > 0.61$, n = 24).

Discussion

We prepared coronal hypothalamic slices containing the SCN at different Zeitgeber times from mice kept under a 12:12 LD cycle. From these slices, we recorded the spontaneous electrical activity with stationary extracellular multiunit electrodes. In all experiments, the electrical activity showed high levels during the animals' subjective day and low levels during the night (see Figure 2.1). This is consistent with previous multiunit electrical activity recordings in the SCN using stationary electrodes (Albus et al., 2002; Bouskila and Dudek, 1993; Brown et al., 2006; Gribkoff et al., 1998; Liu et al., 1997a; Mrugala et al., 2000; Prosser, 1998). Electrical activity rhythms of the SCN have also been investigated by sampling the electrical activity rate from many individual neurons for short durations of time. These sampling procedures yielded similar electrical activity patterns of the ensemble, with a peak during the mid-subjective day (Akiyama et al., 1999; Burgoon et al., 2004; Mason and Rusak, 1990; Prosser, 1998; Saeb-Parsy and Dyball, 2003; Soscia and Harrington, 2004). One study showed influences of time of preparation on the phase of the *in vitro* rhythm. In that study, preparation of the brain slices during the animals' dark period was performed under brief (20 - 30s) light exposure, which may have caused the observed phase shifts (Gillette, 1986). Surprisingly, no other studies have systematically analyzed the influence of preparation time on the peak of the electrical activity rhythm.

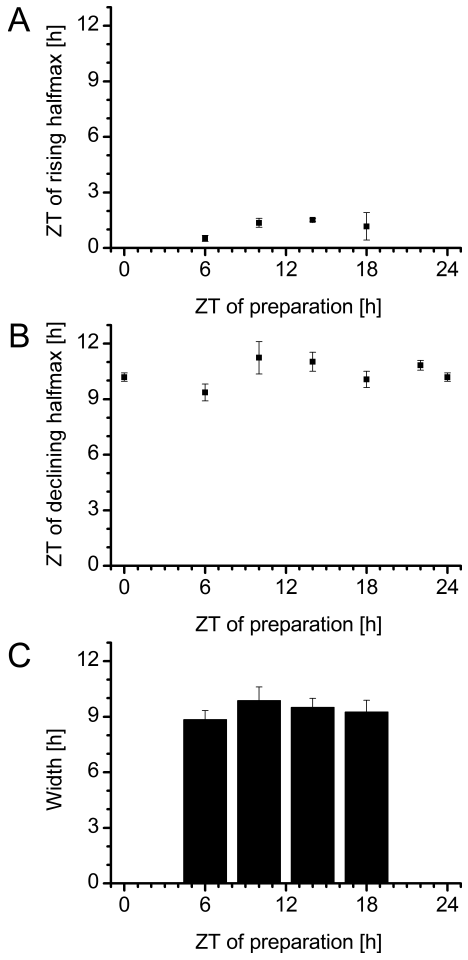


Figure 2.3

Properties of multiunit electrical activity rhythms in the SCN.

(A) Average Zeitgeber time (± SEM) of the half-maximum levels on the rising slope of multiunit electrical activity as a function of time of preparation. No significant differences were observed between preparation times (ANOVA, $p > 0.07$). Note that for some preparation times, the rising phase of the first peak in electrical activity could not be determined.
(B) The average time (± SEM) of the half-maximum levels on the declining phase of the peak as a function of time of preparation. It was consistent for all times of preparation, and no significant differences were found between any of the preparation times (ANOVA, $p > 0.06$). Note that the ZT 0 and ZT 24 data points are repeated and are the same values.
(C) The width of the multiunit peak as a function of time of brain slice preparation. Width of multiunit electrical activity was also unaffected by the time of preparation (ANOVA, $p > 0.61$).

When we obtained two successful recordings from one slice, we averaged the peak times to obtain the best possible estimate for the phase of the SCN as a whole. It is well known that populations within the SCN differ in phase. Simultaneous recordings from populations of

the left and right SCN of the rat can show phase differences of up to 4h, with a mean difference of about 1h (Schaap et al., 2001; Schaap et al., 2003). Populations from the dorsal and ventral rat SCN show mean phase differences of 0.9h (Schaap et al., 2003). Regional differences have also been observed in the mouse SCN (VanderLeest et al., 2007; Brown and Piggins, 2009). Recordings of single mouse SCN neurons in acutely prepared slices show phase differences in Per1 GFP and luciferase expression (Quintero et al., 2003; Yamaguchi et al., 2003). In the present study, we found a range in peak times for each preparation time. Most peaks of the electrical rhythm occurred around mid-day (between ZT 4 - 8), but some were observed outside this range.

We noticed that the data from ZT 2 were influenced by the relatively short interval between slice preparation and determination of the peak time. After preparation at ZT 2, and subsequent settling of the slice for 1h, we started the actual recording at around ZT 3.5. Because of the lack of a clear rising phase at this time of the cycle, a number of peaks could not unambiguously be determined. We concluded that preparation at ZT 2 is not suitable for determination of the first peak, and we performed additional recordings at ZT 0 to obtain data for preparation at the early day. The peak time for preparation at ZT 0 was at ZT 5.19 ± 0.25h (n = 6).

For preparation at ZT 22, the mean peak time occurred relatively early, which was attributable to a very early peak time in four out of the six recorded slices at this preparation time. Light exposure during the beginning of the night causes delays of the circadian pacemaker of the mouse, while light exposure at the end of the night causes advances. As we prepared our slices under dim red light, we wondered whether the short exposure to light may have caused a phase advance. While an exposure time of 3 - 5min of red light may not trigger large behavioral phase shifts, it has been shown that the phase shifting response *in vitro* is larger than the response *in vivo* (Vansteensel et al., 2003b) and is measurable within several hours after slice preparation (Yannielli and Harrington, 2000;

Gillette, 1986). When we repeated the experiments in the dark at ZT 22, using infrared viewers, no differences were detected by ANOVA between any of the preparation times. The obtained peak times at ZT 22, however, were not significantly different from the ones obtained in red light. Therefore, we cannot distinguish whether the early peaks were really due to red-light exposure or to variability in the dataset. The latter would also be in accordance with the finding that no shifts were observed at ZT 14, where phase delays are induced by light. Although the effect is minor or negligible, we suggest that care should be taken with preparations started at ZT 22.

The main focus of our analyses in this article is the peak phase in multiunit electrical activity in the SCN *in vitro*, following preparation at different Zeitgeber times. We also analyzed other parameters of the extracellular multiunit electrical activity peak, such as the phase of half-maximum levels and the width of the peak. Half-maximum levels on the rising slope of the multiunit peak were not significantly different between preparation times, and the half-maximum levels on the falling slope and the width of the peaks were also not different. Taken together, the results show that the waveform of the multiunit electrical activity in the SCN is robust and comparable between preparation times.

There have been a great number of *in vitro* studies in the field of circadian rhythms in which the peak time of the electrical activity rhythm was quantified. The precise time of preparation, however, often has not been provided. To evaluate the time of peak activity observed in the various studies, we restrict ourselves to those studies that provided the preparation time in some detail. Studies performed in rats that were kept on LD 12:12 and were prepared during the light period report average peak times between ZT 5 - 8 (Bergeron et al., 1999; Gillette, 1986; Gillette and Prosser, 1988; Liu and Gillette, 1996; McArthur et al., 1991; McArthur et al., 2000; Prosser, 1998; Rangarajan et al., 1994; Shibata and Moore, 1993). Hamsters housed under a 14:10 LD cycle, also prepared during the light period, showed a narrow range of peak times between ZT 6 and ZT 7 h (Biello et al.,

1997; McArthur et al., 2000; Schak and Harrington, 1999; Yannielli et al., 2002). No phase shifting effects were found in hamsters following preparation at different circadian times (Yannielli and Harrington, 2000). In mice, slice preparation during the second half of the day (ZT 6 - 12) resulted in a peak in SCN activity at ZT 6.3h (Soscia and Harrington, 2004) and when prepared at ZT 2 - 4.5 peaks were observed at ZT 6.8h (Liu et al., 1997a). Multiunit recordings performed in brain slices from mice kept in constant darkness and prepared at circadian time 2 (CT 2) showed activity peaks at CT 5.4h (Albus et al., 2002). Despite differences in techniques, strains, and species, the range of peak times is remarkably narrow. Studies in which the preparation time was provided in a more general way (i.e., preparation was performed during the day) also yielded very similar peak times (for a review, see Gillette, 1991). Together, the findings of these studies indicate a relative robustness in the time of the electrical activity peak *in vitro*.

At the molecular level, the Per1 gene is involved in translational-transcriptional feedback (Albrecht et al., 1997; Yamazaki et al., 2000). Transgenic rats that carry a firefly luciferase gene that is under control of the mouse Per1 promoter (Per1-luc rats) can be used to follow the circadian expression of Per1 both *in vivo* (Yamaguchi et al., 2001) and *in vitro* (Yamazaki et al., 2000). Studies on Per1-luc expression recordings from the SCN show inconsistent results with regard to the effect of preparation time on the phase of the circadian rhythm. Yamazaki et al. (2000) showed no difference in the phase of the rhythm in bioluminescence in the SCN when prepared at ZT 3 or ZT 9. Preparation of slices, with an interval of 2h, showed no consistent effects on the phase of Per1-luc expression rhythm, although a large range in peak times was observed (Abraham et al., 2005). Yoshikawa and coworkers (2005), however, observed consistent delays, of up to 6h, for preparations during the animals' dark phase and at the start of the light period (ZT 1, 17, or 23) (Yoshikawa et al., 2005). No visible light was used when preparations

were started during the dark period of the animal, which suggests that the observed shifts were not induced by light.

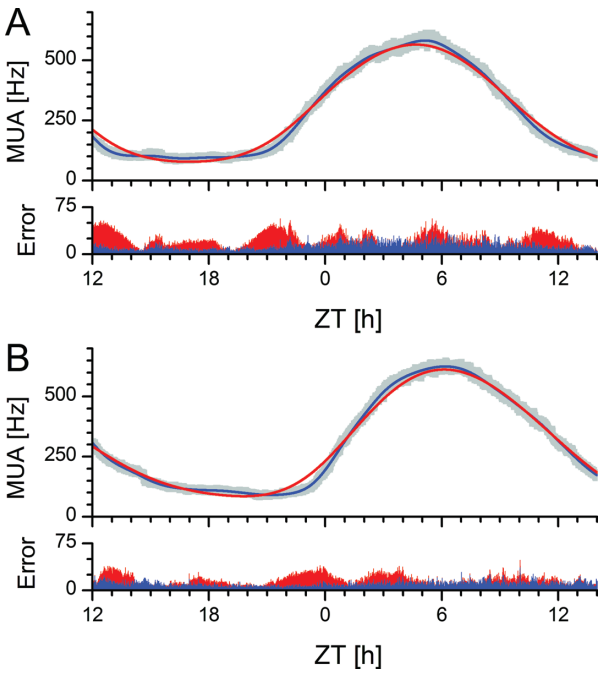
It is unlikely that species differences explain the differences in results, as electrophysiological recordings show comparable peak times for rats, mice, and hamsters. One possibility could be that the Per1 expression rhythm may not always parallel the electrical activity rhythm but may dissociate from it as a consequence of the preparation procedure. Differences between electrical activity and Per1-luc expression rhythms were found in one study that compared Per1 luciferase activity with electrical activity following a phase shift of the LD cycle (Vansteensel et al., 2003b). It is possible, therefore, that slice preparation induced shifts in Per1 bioluminescence rhythms, but no shifts in electrical activity. Given the difference in results between studies on Per1 expression, it is possible that the phase of the Per1 rhythm is more susceptible to phase shifting effects than the electrical activity rhythm.

Our data do not imply that the characteristics of the electrical activity rhythm measured *in vitro* are similar to those *in vivo*. In fact, a number of differences have been observed (Meijer et al., 1997). Some of these differences may be due to the absence of incoming pathways in the slice preparation. In our study, the slices are 400μm thick, while the SCN in mice is ~600μm from the anterior to the posterior side (Abrahamson and Moore, 2001). As a consequence, connections within the SCN are disrupted to some extent by slicing. An analysis of *in vivo* versus *in vitro* characteristics revealed that the *in vivo* autocorrelation of electrical impulses is lower than *in vitro*, leading to an increment in variability *in vivo*. Part of this may stem from incoming information from sleep and activity regulatory areas, as both have been shown to alter SCN electrical impulse frequency (Deboer et al., 2003; Schaap and Meijer, 2001; Yamazaki et al., 1998). In addition, it has been observed that the phase of the SCN rhythm *in vitro* shifts to a larger extent than the SCN *in vivo* (Vansteensel et al., 2003b; van Oosterhout et al., 2008). Furthermore, the electrical

activity peaks *in vivo* are somewhat broader (~2h) than those observed *in vitro* (VanderLeest et al., 2007).

In the present study, we investigated whether the time of preparation affects the phase of the rhythm in the electrical activity in the SCN *in vitro*, and, thus, whether results from different preparation times are comparable. This question is important, as different experimental procedures require different preparation times. We conclude that the peak of the electrical activity rhythm of the SCN renders a robust marker for circadian phase, especially when preparation during the subjective night is performed in darkness, and that the time of the peak is consistent between preparation times.

Supplemental Data



Supplemental Figure 2.1

Effect of applying a higher smoothing parameter

(A, B) Two examples of extracellular multi unit activity plotted as a function of Zeitgeber Time with on the lower axis the deviation of the smoothed data from the raw data (in Hz). Raw data is indicated as grey dots. Weak smoothing (blue line, $\lambda = 1 \times 10^{9}$) provides a low error and a good form estimate of the raw data, i.e. the smoothed line stays within the range of raw data points. Application of a higher smoothing parameter (red line, $\lambda = 1 \times 10^{11}$) introduces a larger error and the shape deviates from the raw data, i.e. at some parts of the curve it is outside the range of the raw data and it renders a more symmetrical peak. Application of a higher smoothing parameter however did not significantly influence the determined time of maximum activity (paired t-test, $p>0.22$).