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*Letter to Neuroscience*

# PHASE DIFFERENCES IN ELECTRICAL DISCHARGE RHYTHMS BETWEEN NEURONAL POPULATIONS OF THE LEFT AND RIGHT SUPRACHIASMATIC NUCLEI

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**The circadian pacemaker of the suprachiasmatic nuclei is a complex multioscillator system, which controls circadian and seasonal rhythmicity (Pittendrigh and Daan, 1976; Meijer et al., 1989). A number of clock genes have been identified that play a key role in the generation of circadian rhythms. These clock genes are expressed in a circadian manner as has been shown in mice, rats and hamsters. The time at which their expression reaches peak values differs among the several genes (Lowrey et al., 2000; Daan et al., 2001; Reppert and Weaver, 2001). Expression profiles for a specific gene may also differ among subdivisions of the suprachiasmatic nuclei. It has been shown that *mPer1* peaks slightly out of phase in the left and right suprachiasmatic nuclei and that the rhythm in *c-fos* expression is significantly different between the dorsomedial and ventrolateral regions (Yamazaki et al., 2000; Schwartz et al., 2000). In the special case that the animal shows splitting of its locomotor activity pattern, *mPer1* in the left and right suprachiasmatic nuclei appeared to oscillate in antiphase (de la Iglesia et al., 2000). Whether the molecular organization within the suprachiasmatic nuclei plays a role in seasonal rhythmicity, allowing animals to track daylength and become reproductive at the proper phase of the annual cycle, receives increasing interest (Daan et al., 2001; Hastings, 2001). The differences in peak expression times that exist between different genes, and the spatial differences in peak time for single genes, are suggestive of a genetic mechanism underlying the multioscillator structure. It is unknown, however, whether phase differences that are observed at the molecular level exist at the level of electrical activity rhythms in the suprachiasmatic nuclei in order to become potentially functional.**

**In this study we investigated the presence of phase differences in neuronal discharge rhythms in the suprachiasmatic nuclei of the rat. To this purpose we combined simultaneous electrophysiological recordings of neuronal populations in the left and right suprachiasmatic nuclei with a detailed analysis of the phase relationship between them. The results demonstrate that neuronal subpopulations of the suprachiasmatic nuclei show phase differences both in their peak and half-maximum times of up to 4 h. We propose that these phase differences may play a role in the plasticity of the circadian timing system.** © **2001 IBRO. Published by Elsevier Science Ltd. All rights reserved.**

Multiunit activity rhythms were recorded in 24 slices. In all slices, peak neuronal activity occurred during the subjective day, and activity troughs during subjective night. In 21 slices, multiunit activity was obtained simultaneously from the left and right suprachiasmatic nuclei (SCN) for one circadian cycle. In the other three experiments, activity was recorded in only one nucleus. In 16 slices, we were able to record a peak also on the second recording day. The peaks occurred during mid-subjective day at Zeitgeber time (ZT) 6.6 ± 0.2 h (mean ± S.E.M.; $n = 37$).

Analysis of multiunit discharge patterns revealed that peak values of firing rate recorded from populations of the two SCN nuclei were out of phase. These phase differences were observable in the raw multiunit traces (Fig. 1). The average phase difference was 0.65 h ± 0.09 h ($n = 21$) on day 1, and 0.52 h ± 0.11 h ($n = 16$) on day 2 (Table 1). There was no significant difference in the magnitudes of the phase differences between day 1 and 2. The population that was relatively delayed on the first day was also delayed on the second recording day in 11 out of 16 of the cases (Fig. 2). In six out of 10 examined cases, peak activity in the left nucleus preceded that of the right one, while in the other four slices peaks occurred in the opposite order. We have no indication for phase differences in the order of 8 h, as has been

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*Abbreviations:* SCN, suprachiasmatic nuclei; ZT, Zeitgeber time.

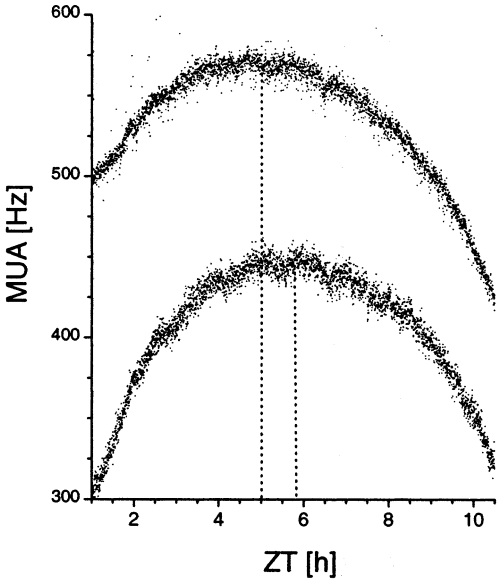


Fig. 1. Multiunit activity (MUA) patterns obtained from the two SCN of one animal. Each trace represents the data from one of the two nuclei. Individual sampled points are plotted (dots). These samples were taken every 10 s. The time of maximum firing rate, which was determined using strongly smoothed data ($\lambda = 10^{11}$), is indicated by a dashed line. For better visualization of the two separate traces, the lower trace was scaled by a factor 0.6. ZT 12 corresponds to light off and ZT 24 to light on.

observed in horizontal slices in hamsters (Jagota et al., 2000).

The question arises whether the observed differences in phase are a consequence of artifactual variability or whether they reflect true physiological differences in phase of the recorded population of neurons. Noise in the data causes random errors in the estimated positions of the peaks. We used Monte Carlo simulations to estimate the possible size of the errors (Fig. 3). The neuronal firing pattern was simulated by a truncated sine, to which low-frequency noise was added. The noise was scaled to an amplitude equal to that of the measured residuals after smoothing of the measured data. The amplitude was computed as the mean difference between maximum and minimum of the residuals in 24 slices.

We simulated 50 000 experiments and computed the difference between two peak positions. The phase differences were randomly divided into groups with a size equal to the sample size of the measured differences. The differences in each group were averaged, resulting in more than 1000 averaged differences. This produced a frequency distribution of errors (Fig. 3). The 99.9th percentile of this distribution was 0.23 h ($n = 21$) or 0.24 h ($n = 16$). Our results (0.65 h on day 1 and 0.52 h on day 2) lie far outside this area. We conclude that the $P$ values are below 0.001. The low-frequency noise was obtained by smoothing of white noise. We found that the width of the error distribution depends on the amount of smoothing. To be conservative, we took the value at which the widest distributions occurred.

The phase differences appeared significant when they were tested against zero with a $t$-test ($P < 0.0005$).

Table 1. Phase differences between populations of neurons (conservative analysis)

| | Day 1 | Day 2 |
|---|---|---|
| Range (h) | 0.09–1.74 | 0.02–1.44 |
| Mean (h) | 0.65 | 0.52 |
| S.D. (h) | 0.43 | 0.45 |
| S.E.M. (h) | 0.09 | 0.11 |
| $n$ | 21 | 16 |
| Significance | $P < 0.001$ | $P < 0.001$ |

Because the $t$-test probably underestimates the error distribution, the Monte Carlo simulations produce a more reliable estimate of the significance of the phase differences (Fig. 3). We evaluated the effects of the smoothing, by calculating the phase differences after minimal smoothing of the data ($\lambda = 10^3$), thereby allowing ultradian and higher-frequency variations to affect the estimated peak time. Minimal smoothing resulted in phase differences of $1.15 \pm 0.19$ h (range: 0.01–4.77 h; $n = 21$) on day 1, and $1.13 \pm 0.31$ h (range: 0.04–5.12 h; $n = 16$) on day 2.

The results were also verified using another phase marker, the time at which the multiunit activity reaches half-maximum. This phase marker was calculated on more strongly smoothed data ($\lambda = 10^{11}$), on the declining slope of the curves. This procedure resulted in phase differences of $0.99 \pm 0.23$ h (range: 0.03–4.68 h; $n = 21$) on day 1, and $0.42 \pm 0.10$ h (range: 0.03–1.41 h; $n = 16$) on day 2.

Our data reveal that in acutely prepared slices, neuro-

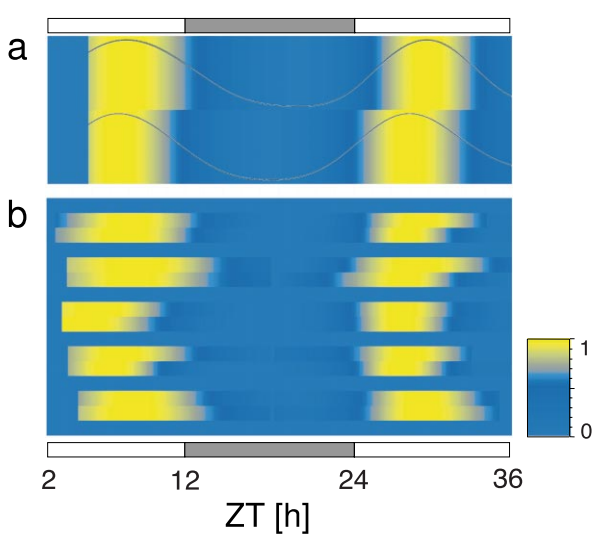


Fig. 2. Multiunit activity (MUA) patterns in contralateral SCN. Multiunit data were smoothed and normalized for each day. The normalized multiunit data are also represented by color codes for better visualization of the data. The bars above the figures indicate (extrapolated) light:dark cycles to which the animals had been entrained. ZT 12 corresponds to light off and ZT 24 to light on. (a) In this example the normalized multiunit patterns recorded in two sides of the SCN are plotted above each other. (b) Additional examples are shown in which bilateral multiunit activity was recorded successfully for 2 days. The upper four recordings show multiunit rhythms in which the same population was advanced on both day 1 and day 2. In the fifth recording, the populations were in phase on both recording days.

nal populations from the left and right SCN express circadian rhythms that are out of phase. The phase differences were observed when the peak in multiunit activity was determined after strong smoothing and after minimal smoothing of the data. Moreover, phase differences were apparent when a different phase marker was used, i.e. the time of half-maximum discharge rate. Although the latter is mathematically appealing as it provides a sharper transition point, the data indicate that the variability in the declining phase of the oscillations was actually larger than the variability in the measured peak levels. We attribute this to the differences that appeared to exist in the widths of the peaks. Nevertheless, we regard this analysis as support for our conclusion that true phase differences exist between populations of SCN neurons.

Minimal smoothing of the data resulted in phase differences ranging from 0.01 to 4.77 h, whereas strong smoothing resulted in phase differences of 0.09–1.74 h (on the first day). It is difficult to assess which of these procedures offers the most reliable estimate of the phase differences. For one reason, the circadian discharge pattern of the SCN is characterized by additional ultradian components (Meijer et al., 1997) and oversmoothing may lead to underestimation of true differences. On the other hand, minimal smoothing does not exclude the possibility that an artifactual change in discharge level contributes to a measured phase difference. We conclude that by strong smoothing of the data we have obtained a conservative estimate of phase differences between the two neuronal populations, but it is not necessarily a more reliable measure.

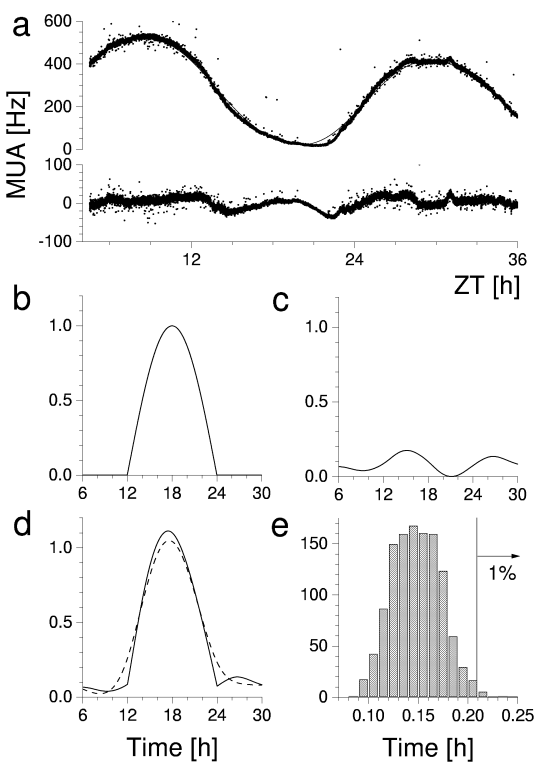


Fig. 3. Monte Carlo simulations for the determination of significance of phase differences. (a) In the upper trace a multiunit activity (MUA) pattern is shown (dots) with its smoothed curve (line). In the lower trace the residuals are plotted. (b) For the simulations, the basic waveform of the multiunit rhythm was simulated by a truncated sine. (c) Variation was introduced by adding noise that was colored by filtering out high-frequency components. The amount of noise was equal to the residuals obtained after smoothing the multiunit recordings. (d) The colored noise was added to the sine curve (straight line). These data were smoothed with the same parameter that was used for the physiological data and resulted in a curve indicated by the dashed line. (e) The phase difference between peak times was calculated and the average over 21 simulations was computed. This procedure was repeated ($>1000$ times) to obtain a frequency distribution of the averaged phase differences. The 99th percentile of this distribution is 0.21 h. Thus, our averaged phase difference of 0.65 h is highly significant.

The size of the neuronal populations can be estimated on the basis of single-unit recordings in slices. These have indicated discharge rates during peak activity of about 5–8 Hz (Groos, 1982; Gillette et al., 1995; Schaap et al., 1999). We assume therefore that we recorded from about 100 neurons, which is about 1% of one SCN nucleus. The large range in phase differences that were measured in rats that had been kept on a 12-h light–dark cycle indicates that small groups of neurons within the SCN may show considerable phase differences. This suggests that the SCN pacemaker does not oscillate as a whole, but that regional differences may exist with respect to its electrical activity.

Simultaneous recordings from dispersed SCN neurons revealed differing circadian periods and oscillations that were out of phase with one another (Welsh et al., 1995; Liu et al., 1997; Herzog et al., 1998; Honma et al., 1998). In organotypic cultures of the SCN, different phase relationships were observed among neurons, with most neurons oscillating in phase (Shinohara et al., 1995; Herzog et al., 1998). Whether the observed phase relationships arose as a consequence of limited interneuronal communication or as a consequence of the culturing condition remained unclear (Welsh et al., 1995; Herzog et al., 1997). In this respect, measurements in the acutely prepared slice provide a more accurate description of the physiological state of the SCN pacemaker. In two *in vitro* studies, single-unit discharge patterns in the left and right SCN have been compared but led to conflicting results. As these two studies sampled neurons at different locations of the SCN, and from different animals, subtle phase differences between populations in the SCN were probably obscured (Zlomanczuk et al., 1991; Zhang and Aguilar-Roblero, 1995).

In horizontal slices from hamsters, it has been demonstrated that two electrophysiological components become apparent (Jagota et al., 2000) that are not present in coronal slices (Bouskila and Dudek, 1993; Meijer et al., 1997; Gribkoff et al., 1998). These two components were recorded by a single electrode. It is unknown whether the two components reflect different groups of neurons, or whether they are present at the level of the single neuron. Our data contribute to the issue by showing different peak times at different locations within the SCN. This demonstrates that phase differences within the SCN can be generated by separate groups of neurons. It is unknown, however, whether the differences in phase between neuronal oscillations that we describe relate to the two components that emerge in a horizontal slice.

The phase differences that we found are supported by qualitative observations *in vivo*. Multiunit discharge patterns from the left and right SCN revealed phase differences in a minority of cases (three out of 12 animals) (Inouye and Kawamura, 1982). While a quantitative analysis would allow a more precise comparison with our data, the phase differences were of a sufficiently large magnitude to be noticeable by eye. The occurrence of phase differences *in vivo* suggests that the differences that we found *in vitro* may be present in the intact animal as well.

It has been proposed that the circadian pacemaker of the SCN is composed of two functionally separate oscillators, called the morning and evening components (M and E respectively, see Pittendrigh and Daan, 1976). Evidence for this model is derived from several behavioral characteristics such as: (i) the animal's ability to follow daylength (Pittendrigh and Daan, 1976), (ii) differences in light-induced shifts in onset and offset of behavioral activity and and the evening and morning fall of pineal *N*-acetyltransferase (Illnerova and Vanecek, 1982; Elliott and Tamarkin, 1994; Meijer and De Vries, 1995), (iii) splitting (Pittendrigh and Daan, 1976) and (iv) differences in phase shifting responses of split components (Meijer et al., 1988, 1990).

The interpretation of a multioscillator structure has recently been supported by a number of molecular biological investigations (Takumi et al., 1998; de la Iglesia et al., 2000; Yamazaki et al., 2000; Daan et al., 2001; Hastings, 2001). We have now demonstrated that phase differences are also present in the rhythm of spontaneous action potentials between different neuronal subpopulations. These phase differences render sets of neurons that are potentially able to carry information on daylength on the basis of their mutual phase relationships. Future experiments will have to evaluate this possibility by exploring the observed phase differences as a function of photoperiod.

## EXPERIMENTAL PROCEDURES

The multiunit activity rhythms of SCN neurons were measured as described previously (Meijer et al., 1997). Wistar rats were entrained to a 12-h light–dark cycle. All efforts were made to minimize both the suffering and the number of animals used, and all experiments were carried out in accordance to the European Communities Council Directive of 24 November 1986 (86/609/EEC). Coronal slices were prepared at the beginning of the subjective day and maintained in an interface chamber. The slices contained at least 50% of the rostrocaudal extent of the SCN, and all of the ventrodorsal extent. To ensure that the size of the two nuclei in the slice was comparable, slices were carefully cut perpendicular to the rostrocaudal axis. Extracellular electrical activity of populations of SCN neurons was measured by platinum/iridium electrodes (75 μm diameter) and subsequently amplified and bandwidth-filtered. Spike triggers were used to select action potentials with amplitudes above a certain level. Spikes were counted electronically every 10 s and stored for off-line analysis.

The technique of multiunit recording makes it possible to record the activity rhythm of a population of neurons for more than one circadian cycle (Bouskila and Dudek, 1993; Gribkoff et al., 1998). Multiunit recordings are technically difficult to perform, and very low levels of electronic noise as well as high mechanical stability are required for stable long-term measurements. By using stable manipulators, slice support and fixation of the slice in the interface chamber, we were able to achieve a stable signal for long recording times. Two neuronal populations, one from the left and one from the right nucleus of the SCN, were measured simultaneously with multiunit electrodes.

From these recordings, the time of maximal activity in the individual circadian discharge rhythms was determined and used as a phase marker. In addition we used the half-maximum discharge rate as a second phase marker. Apart from a circadian component, multiunit discharge patterns consist of ultradian components and noise which is either physiological or artifactual (Meijer et al., 1997; Yamazaki et al., 1998; Jagota et al., 2000). Ultradian components and noise can interfere with the determination of the peak times. The human eye can easily smooth the data and indicate probable positions of peak activity (Fig. 1). Simply seeking the highest value in the series of numbers will not work, because of the noise and ultradian variation.

To minimize the noise and ultradian components the data were smoothed with penalized least squares. Let a series of multiunit data be indicated by $y_i$, $i = 1...m$, and let $z_i$ be a corresponding smooth series. We minimize the following function:

$$Q = \sum_{i=1}^{m} (y_i - z_i)^2 + \lambda \sum_{i=3}^{m} (\Delta^2 z_i)^2$$

where $\Delta^2 z_i = z_i - 2z_{i-1} + z_{i-2}$ indicates second differences. The first term of $Q$ is a measure of fit of $z$ to the data $y$, the second a penalty on the roughness of $z$. By increasing $\lambda$, a smoother $z$ will be obtained. This smoother has a long history and goes back at least to Whittaker (1923) (see Eilers, 1994; Eilers and Marx, 1996, for details and other applications). The smoothing spline is very similar to this approach, but unnecessarily complicated, while regression smoothers (like B-splines) pose the difficult problem of knot optimization. For our application it is also important that this smoother does not introduce phase shifts and has no unpleasant boundary effects (like kernel smoothers or Fourier filters). We chose $\lambda = 10^{11}$ on the basis of visual inspection. With this amount of smoothing we effectively have a zero-phase low-pass filter with a 12-dB/octave slope and a corner frequency of approximately 1 cycle per 10 h.

Differences between peak times of the two populations were analyzed for statistical significance. We are not aware of a model that adequately predicts the effect of noise or high-frequency variability on peak times. Therefore, Monte Carlo simulations were performed to determine significance levels. In these simulations, the basic multiunit pattern was represented by a truncated sine, as an adequate description of multiunit waveform peaks. Variability was introduced by adding noise of the same magnitude as the residuals in our experimental data (see above). We analyzed the sum of sine and noise in the same way as the measured data. The cumulative distribution of the results was used to obtain the significance levels. The results were considered statistically significant when the probability level was less than 0.01.

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