Current Biology, Vol. 12, 1130–1133, July 9, 2002, ©2002 Elsevier Science Ltd. All rights reserved. PII S0960-9822(02)00923-5

Cryptochrome-Deficient Mice Lack Circadian Electrical Activity in the Suprachiasmatic Nuclei

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Summary

The mammalian master clock driving circadian rhythmicity in physiology and behavior resides within the suprachiasmatic nuclei (SCN) of the anterior hypothalamus [1–2]. Circadian rhythms are generated by a set of clock genes via intertwined negative and positive autoregulatory transcription-translation feedback loops [3–6]. The *Cryptochrome 1* and *2* genes are indispensable for molecular core oscillator function, as evident from the arrhythmic wheel-running behavior and lack of rhythmic clock gene expression in *mCry1/mCry2* double-mutant mice in constant darkness [7–10]. In the present study, using real-time multiunit electrode activity recordings in hypothalamic slices, we show that SCN neurons from *mCry*-deficient mice kept in constant darkness lack circadian oscillations in firing patterns. This proves that *cryptochromes,* and thus an intact circadian clockwork, are prerequisites for circadian electrical activity in SCN neurons. Interestingly, when *mCry*-deficient mice were kept in normal light-dark conditions and SCN slices were prepared 2 hr after the beginning of the day, a single noncircadian peak in neuronal activity was detected. This light-induced rise in electrical activity of the SCN may explain why *mCry*-deficient mice lack the arrhythmic short bouts of wheel-running activity and instead show apparently normal behavior in normal day-night cycles.

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Results and Discussion

The *mCry*-Deficient SCN Lacks Circadian Rhythms in Electrical Activity

To assess whether a functional molecular oscillator is necessary to drive circadian electrical activity in the SCN, we performed multiunit electrode activity (MUA) recordings on SCN slices from wild-type and clock-deficient *mCry1/mCry2* mutant mice housed in cages equipped with a running wheel to monitor behavioral activity. In a first set of experiments, mice were kept in constant darkness (DD) and were sacrificed at a time corresponding to the beginning of the subjective day of wild-type animals (around circadian time 2, CT 2, as deduced from the activity patterns). Electrical activity measurements were started within 1 hr after slice preparation. Wild-type SCN slices exhibited a marked circadian rhythm of multiunit activity that could be recorded over two consecutive cycles (Figure 1A). Peaks of firing frequency were observed at CT 5.42 $\pm$ 1.18 hr ($n = 7$, mean $\pm$ SD) and 2.23 $\pm$ 1.58 hr ($n = 7$) on the first and second day of recording, respectively. The mean duration between the two peaks (20.82 $\pm$ 1.80 hr, $n = 7$) was significantly shorter than the near 24-hr period of free running-wheel activity (23.77 $\pm$ 0.07 hr [7], $p < 0.05$).

Electrical recordings in SCN slices from *mCry*-deficient mice ($n = 4$) all showed an initial high level of firing that rapidly decreased during the first hours of recording (Figure 1B). Although this electrical behavior is in marked contrast to the increasing frequency observed in wild-type SCN slices, it indicates that the lack of mCRY proteins does not interfere with the ability of SCN neurons to generate action potentials. Neuronal discharge activity then stabilized at a mid-to-low plateau level and dropped to low levels after 20–30 hr. During recording, we never observed any reappearance of high-frequency electrical activity. From these data, we conclude that the presence of cryptochromes, and thus an intact circadian clockwork, is necessary to achieve a circadian rhythm in membrane excitability of SCN neurons.

The Electrical Activity of the *mCry*-Deficient SCN Is Affected by Light

The behavior of *mCry*-deficient mice under normal light-dark (LD) cycles strongly resembles that of light-entrained wild-type animals. Instead of the short intervals of wheel-running activity and rest, animals display a rather long period of activity (see [7]; also compare actograms in Figures 1B and 2B). This prompted us to also conduct electrophysiological experiments on brain slices from wild-type and *mCry*-deficient mice that had been kept in a 12:12 light-dark cycle. The multiunit activity of light-entrained wild-type SCN slices, isolated at the beginning of the light phase (ZT 2; zeitgeber time 2), displayed high levels of firing during the subjective day that dramatically decreased during subjective night and reappeared in the following subjective day (Figure

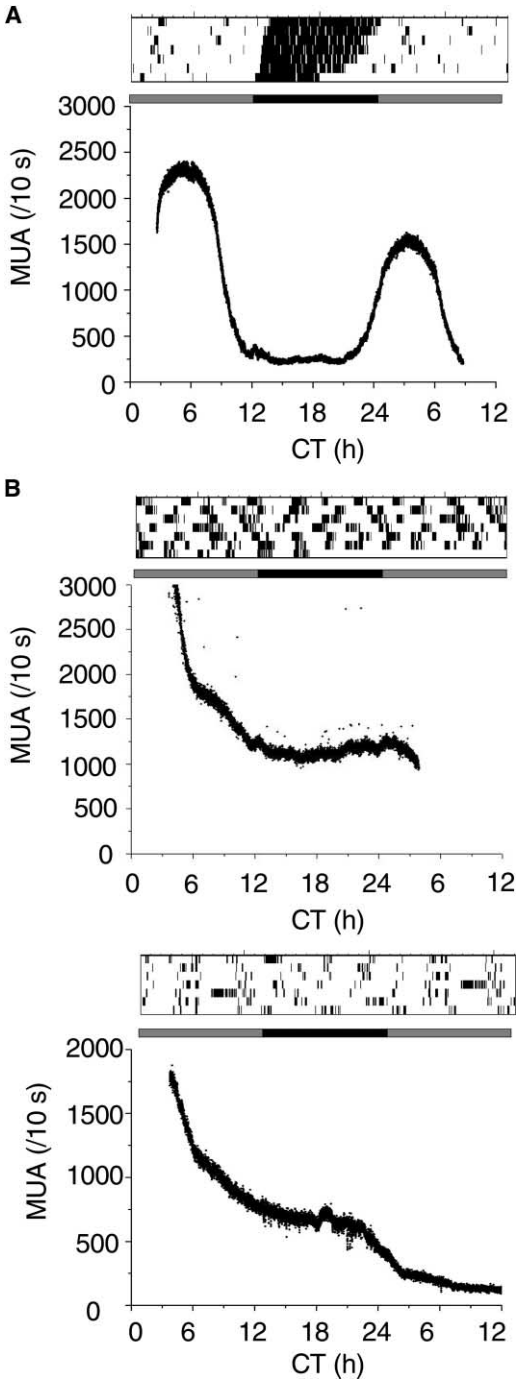

Figure 1. Firing-Rate Patterns in SCN Slices from Wild-Type and *mCry*-Deficient Mice Kept in Constant Darkness (DD)

The running-wheel activity patterns of the mice in the last 7 days prior to slice preparation are indicated above the records. The black and gray bars above each plot indicate the subjective night and day, respectively. CT, circadian time.

(A) Circadian rhythm in multiunit activity (MUA) in the SCN of a wild-type mouse.

(B) Representative examples of the gradually decreasing firing frequency in the SCN of a *mCry1*−/−*mCry2*−/− mouse.

2A). Peak levels were reached at ZT 6.72 ± 0.91 hr on day one and ZT 3.78 ± 0.76 hr on day two. The peak interval (21.04 ± 0.99 hr, n = 9) was significantly shorter than the 24-hr entrainment period ($p < 0.05$) but was comparable to that from mice in DD ($p = 0.76$).

SCN slices from *mCry*-deficient mice that were kept under the same LD conditions and sacrificed at the same time as the wild-type animals (ZT 2) showed an initial sustained decrease in firing (Figure 2B). This initial pattern was similar to that observed in slices from animals kept in DD conditions (Figure 1B). Strikingly, the discharge rate of mutant SCN stopped decreasing at the end of the subjective day and rose to a maximum at mid-subjective night (CT 15.34 ± 4.59 hr, n = 5). This peak in firing rate reached discharge levels resembling those obtained in the wild-type SCN ($p = 0.15$). However, the circadian discharge pattern as a whole was significantly smaller in amplitude than the one measured in wild-type SCN slices. The trough value represented 47% ± 19% and 10% ± 7% of the peak value in mutant and wild-type SCN, respectively. Importantly, this pattern of neuronal firing did not reappear in the next 24 hr (Figure 3), suggesting that it is not due to an intrinsic rhythmic property of the SCN.

The peak of firing observed during the first subjective night in mutant SCN might be caused by either the preceding LD cycle or the slice preparation procedure. To address this issue, we repeated the experiment under conditions in which animals were sacrificed at ZT 14 instead of ZT 2. Under these conditions, we could not detect any peak in the electrical discharge pattern (n = 2). Instead, we observed a rapid decrease in multiunit activity during the first 3–4 hr of recordings. Then, the discharge rate stabilized at a plateau of mid-to-low activity for about 24 hr (Figure 2C). Hence, the procedure of slice preparation cannot account for the peak of firing observed in mutant SCN prepared at ZT 2. From these data, we conclude that the peak in electrical activity in SCN slices from *mCry*-deficient animals kept on a LD cycle was induced by the light exposure immediately before slice preparation.

Interestingly, a light pulse is able to induce *mPer1* and *mPer2* expression in the SCN of *mCry1/mCry2* double-mutant mice [9]. Moreover, whereas under LD conditions *mPer1* transcript levels in the SCN of *mCry*-deficient mice are constitutively high, the level of *mPer2* mRNA is more than 2-fold higher at ZT 6, as compared to ZT 18 [8]. This suggests that rhythmic *mPer2* expression might be imposed by the LD cycle rather than by an endogenous circadian oscillator in the SCN of *mCry1*−/−*mCry2*−/− mice. We hypothesize that, in the absence of cryptochromes, the environmental LD cycle could act as a daily trigger, provoking a kick-start of the remaining molecular clockwork. In this scenario, the induction of *mPer* genes by light at ZT 0–2 (slices were prepared at ZT 2) may have been sufficient to directly or indirectly provoke a peak of the neuronal firing. In the absence of a new stimulation ex vivo, the system is not able to start a new cycle, as shown by our results. Assuming that this model also holds for the in vivo situation, such light-induced electrical activity in the SCN could be responsible for the almost normal behavioral activity pattern of *mCry*-deficient animals in light-dark cycles.

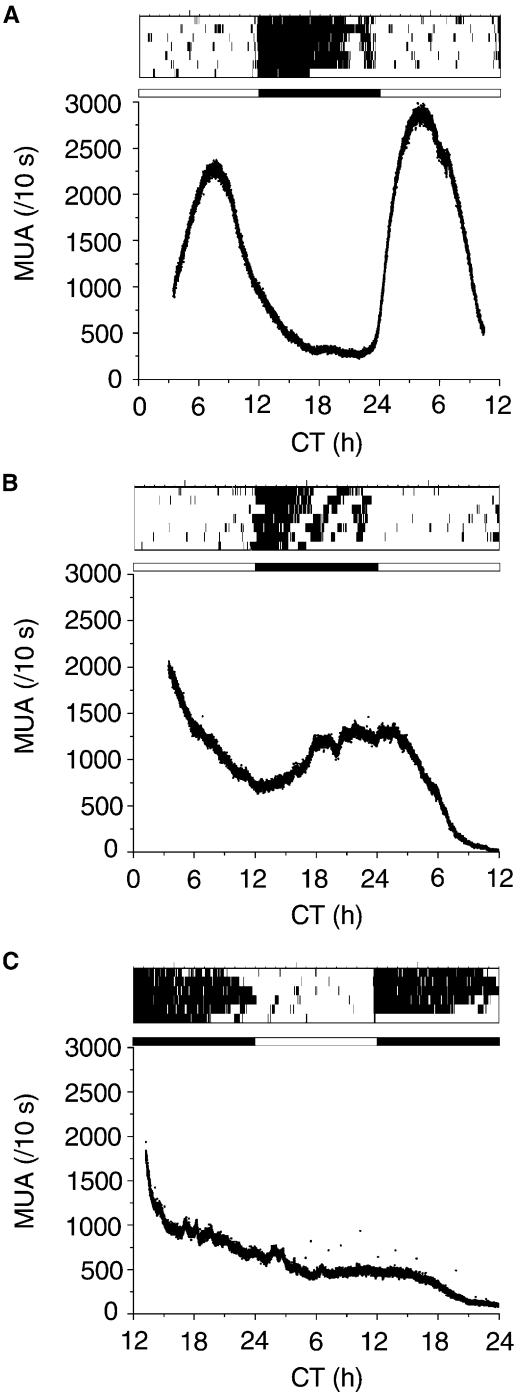

Figure 2. Firing-Rate Patterns in SCN Slices from Wild-Type and *mCry*-Deficient Mice Kept in a Light-Dark Cycle

Running-wheel activity patterns prior to slice preparation are indicated above the records. The black and white bars above each plot indicate the previous light-dark cycle.

(A) Circadian electrical activity in wild-type SCN.

(B) In *Cry*-deficient SCN prepared at ZT 2, the firing rate increased during subjective night.

(C) When prepared at the beginning of the dark phase, the slice did not exhibit any peak of firing during subjective day or subjective night.

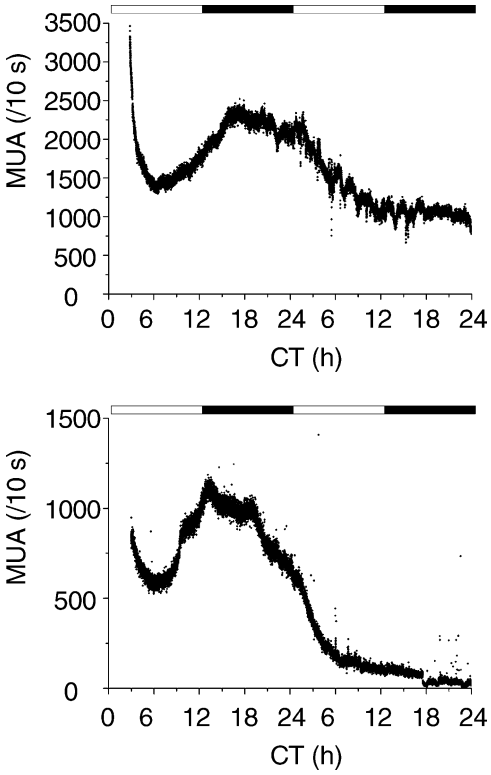

Figure 3. Long-Term Recordings in SCN Slices from *mCry*-Deficient Mice Kept in a Light-Dark Cycle

Slices were prepared at ZT 2. Note that the light-induced peak in electrical activity does not appear during the second day of recording.

Conclusions

So far, no direct evidence had been provided that the molecular clockwork is an absolute necessity to drive circadian rhythms in the discharge rate of SCN neurons. In dispersed SCN neurons from *Tau* mutant hamsters, the average period length of electrophysiological rhythms correlates well with the circadian periodicity in behavior [11]. A similar parallel between behavior and electrophysiology was observed in homozygous *Clock* mutant mice. Animals rapidly became arrhythmic in constant darkness [12], whereas dispersed SCN neurons show a near-24-hr rhythm in electrical activity for 2–3 days before turning into arrhythmicity [13]. In marked contrast, cultured organotypic slices of homozygous *Clock* mutant mice have recently been shown to still exhibit a circadian rhythm in SCN electrical activity, which was attributed to the presence of intact intercellular communication in the slices [14]. Our finding that acutely prepared SCN slices of the clock-deficient *mCry1/mCry2* double-mutant mice, despite the relative integrity of intercellular networks, are electrically arrhythmic provides direct genetic evidence that cryptochromes, and thus an intact circadian core oscillator, are indispensable for circadian rhythmicity in electrical activity in the SCN. We are currently exploring the intriguing possibility that light, to some extent, can normalize the behavior of *mCry*-deficient mice.

Experimental Procedures

Animals

Wild-type and *mCry1*−/−*mCry2*−/− mice (C57Bl6/Ola129 hybrid) of either sex were used (6–24 weeks). Genotypes were assessed by Southern blot analysis of tail DNA as previously described [7]. All animals received food and water ad libitum and were housed individually in cages (type 2) equipped with a running wheel (12 cm diameter). They were entrained to a 12:12 light-dark cycle and then released in constant darkness when necessary. A homemade computer system registered the presence or absence of voluntary wheel running each minute. Experiments were performed according to the principles of laboratory animal care.

Multiunit Activity Recording

Mice were killed by decapitation, and, after rapid dissection, the brain was cooled down in ice-cold recording solution (see below) and trimmed. Animals kept under DD conditions were handled under dim red light until the optic nerve was cut. Coronal hypothalamic slices (400–600 μm) containing the paired SCN were made using a tissue chopper. Slices were transferred to the thermostated (36°C) recording chamber and were continuously perfused with a solution containing 116 mM NaCl, 5 mM KCl, 1 mM NaH_2PO_4, 1 mM $MgSO_4$, 2 mM $CaCl_2$, 26 mM $NaHCO_3$, 10 mM glucose, and 5 mg/l gentamycin and were saturated with 95%O_2/5%CO_2. Recordings started after 30–60 min of recovery. Extracellular electrical activity was measured by platinum/iridium electrodes (75 μm diameter) and was subsequently amplified and bandwidth filtered. Action potentials were detected by spike triggers, counted electronically every 10 s, and were stored for off-line analysis [15]. Recordings lasted for at least 24 hr and for up to 50 hr. One electrode was implanted in the left SCN, and the other in the right SCN, rendering two recordings per slice. Data from the left and right SCN were always similar, and means per slice were calculated for data analysis.

The time scale of electrical recordings was based on the previous light regime to which the animals were exposed. Therefore, recordings after LD conditions refer to zeitgeber time (ZT), with ZT 12 defined as light offset. Recordings after constant darkness refer to circadian time (CT), with CT 12 defined as the onset of running-wheel activity of wild-type animals. Since *Cry*-deficient mice are arrhythmic in DD, CT was determined according to their wild-type counterparts.

Data Analysis

Peaks of firing were determined after strong smoothing of data. Values obtained from both electrodes in the same preparation were averaged. All experiments were then considered independently of one another. All values are expressed as mean ± SD. Data were compared with Student's unpaired t tests.

Acknowledgments

We thank Dr. W.J. Schwartz for his useful comments on our manuscript and M. van Steensel for her help with the preparation of the figures. We thank J. Schaap and Dr. F. Tamanini for discussion and H. Duindam and J. Janse for technical assistance. This work has been supported by a Marie Curie Fellowship of the European Community program "Improving Human Research Potential and the Socio-economic Knowledge Base" under contract number HPMF-CT-2000-00950 to X.B., a NWO-Hungarian cooperation grant to J.H.M., and a Spinoza Premium of the Netherlands Organization for Scientific Research (NWO).

Received: April 2, 2002
Revised: May 7, 2002
Accepted: May 7, 2002
Published: July 9, 2002

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