Constant light desynchronizes mammalian clock neurons

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Circadian organization can be disrupted by constant light, resulting in behavioral arrhythmicity or 'splitting' of rhythms of activity and rest. By imaging molecular rhythms of individual clock neurons in explanted mouse clock nuclei, we now find that constant light desynchronizes clock neurons but does not compromise their ability to generate circadian rhythms. Cellular synchrony within clock nuclei is disrupted during arrhythmicity, whereas neurons in the left and right clock nuclei cycle in antiphase during 'splitting.'

Neural circadian clocks are composed of populations of circadian pacemaker neurons organized to drive coherent daily rhythms in behavior and physiology. An unresolved question is how external stimuli that disrupt behavioral and physiological rhythmicity (such as shift work and jet lag in humans) affect the function and organization of central neural pacemakers. Exposure to constant light (LL) disrupts overt rhythms and induces circadian arrhythmicity in mammals[1] and other species. Two general hypotheses have been advanced to explain this phenomenon: light either stops the cell-autonomous molecular oscillations that generate circadian rhythms, or it desynchronizes the ongoing rhythms of the individual oscillator neurons that make up neural circadian clocks. We have now successfully tested these hypotheses in the mammalian brain circadian clock and have examined the cellular basis for the 'splitting' of behavioral rhythms into two bouts of activity and rest per 24-h interval as occurs in some individuals exposed to LL[1].

For this study we used *Per1*:*GFP* transgenic mice in which rhythmic activation of the Period1 (*Per1*) clock gene promoter can be monitored at the single cell level in real time using time-lapse confocal microscopy[2]. Mice were exposed to LL while their locomotor activity rhythms were assayed by wheel running (see **Supplementary Methods** online for details). Individual mice in LL either (i) became behaviorally arrhythmic (ii) remained rhythmic with lengthened free running period or (iii) showed split locomotor activity rhythms with two bouts of activity per 24-h interval[1]. To study the organization of the biological clock in the behaviorally arrhythmic state, we selected mice that had been rendered behaviorally arrhythmic but that continued to show robust overall levels of activity (**Fig. 1a**, $n = 5$). We then analyzed the *Per1* gene transcription dynamics of their hypothalamic suprachiasmatic nuclei (SCN), which make up the circadian clock for locomotor activity[3].

SCN of arrhythmic mice were dissected and explanted into organotypic culture and their *Per1* promoter–driven GFP fluorescence rhythms recorded during the initial 4 d *in vitro*. SCN *Per1*:*GFP* signals from behaviorally arrhythmic mice showed significant levels of *Per1* promoter activity, as indicated by fluorescence intensities well above background, but SCN circadian rhythmicity was severely disrupted (**Fig. 1b**). The weak oscillations we observed in SCN from arrhythmic mice showed trough-to-peak amplitudes of only 1.09-fold on average (that is, peaks only 9% greater than troughs, ±0.02% s.e.m.), whereas the prominent SCN *Per1*:*GFP* rhythms from mice maintained on normal light-dark (LD) cycles are typically 2- to 3-fold in amplitude[2].

In contrast, imaging of individual cellular rhythms within SCN from behaviorally arrhythmic mice revealed the persistence of robust neuronal circadian rhythms in *Per1* promoter activation that were 1.5- to 3-fold in amplitude, similar to the amplitudes of cellular *Per1*:*GFP* rhythms recorded in SCN from LD mice[2]. The SCN

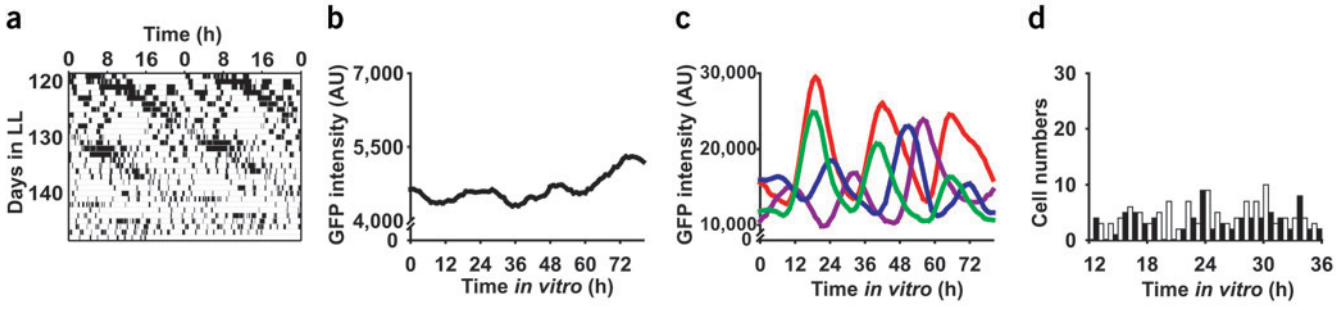

Figure 1 Behavioral and SCN rhythms from an arrhythmic constant light–treated mouse. (**a**) Actogram of wheel running activity. Black marks indicate wheel revolutions. Note loss of temporal organization in latter portion of record. (**b**) Time-lapse SCN *Per1*:*GFP* fluorescence signals for 3.5 d *in vitro*. (**c**) Individual SCN neuronal *Per1*:*GFP* rhythms from SCN in **b**. Four representative cells are plotted for clarity (colored lines). (**d**) Peak time histograms of individual neuronal rhythms. Peak times of neurons in the right SCN are plotted with black bars, whereas those in the left SCN are plotted with open bars. Histograms for this and the following figures are for hours 12–36 *in vitro*. $n = 193$ cells. Animal care and use was reviewed and approved by the Vanderbilt University IACUC.

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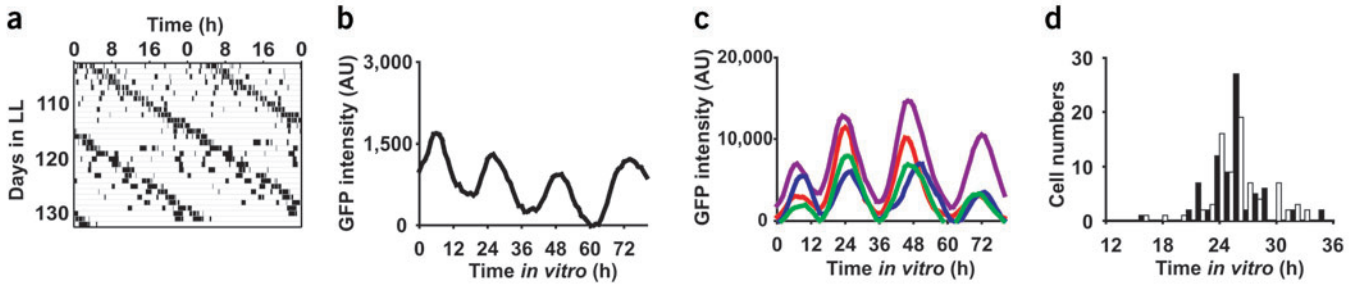

Figure 2 Behavioral and SCN rhythms from a rhythmic constant light–treated mouse. (**a**) Actogram of wheel running activity from a mouse that remained rhythmic in LL. (**b**) Time-lapse SCN *Per1*:*GFP* fluorescence signals. (**c**) Individual SCN neuronal *Per1*:*GFP* rhythms from SCN in **b**. (**d**) Peak time histograms of individual neuronal rhythms. $n = 154$ cells.

neuronal rhythms of arrhythmic LL mice were, however, notably desynchronized in phase (**Fig. 1c**). Peak times of individual neuronal rhythms from arrhythmic mice were widely dispersed (**Fig. 1d**) with a mean standard deviation of 6.1 ± 0.5 h (mean ± s.e.m., throughout) ($n = 10$ nuclei). Similar robust but desynchronized neuronal rhythms were observed in the SCN of all arrhythmic mice assayed ($n = 843$ cells, 10 nuclei, 5 mice).

To test whether SCN cellular desynchronization in LL was specific to behaviorally arrhythmic mice, we also assayed SCN molecular rhythms of mice in which significant behavioral rhythmicity persisted in our LL conditions (**Fig. 2a**, $n = 5$). SCN from rhythmic LL mice showed clear circadian rhythms in gene expression averaging 2.57-fold (± 0.69) in amplitude (**Fig. 2b**), similar in amplitude to SCN rhythms from LD entrained mice[2]. In addition, individual neurons within the SCN of rhythmic LL mice also showed robust *Per1*:*GFP* rhythms, but with a much greater degree of synchrony than those of arrhythmic LL mice (**Fig. 2c**). Peak time histograms of individual cell rhythms from rhythmic LL mice SCN showed the majority of cells synchronized in a peak centered near the projected onset time of the locomotor rhythm, with a few cell rhythms in antiphase to the main cluster (**Fig. 2d**). The standard deviation of cell peak times was significantly less in SCN from LL rhythmic mice than in SCN from arrhythmic mice (3.6 ± 1.0 h, $n = 10$ nuclei, $P < 0.01$), indicating greater phase coherence. Similar results were observed in SCN of all rhythmic LL mice ($n = 881$ cells, 10 nuclei, 5 mice).

Whereas constant light desynchronized the neuronal populations within the SCN of arrhythmic animals, it did not significantly affect the generation and expression of circadian rhythmicity by individual neurons. A high proportion of imaged neurons in SCN from arrhythmic mice were rhythmic (735/843, 87%), similar to LL rhythmic mice (783/881, 89%) and LD mice (89%, ref. 2). In addition, the calculated free running periods of individual neurons in SCN from arrhythmic LL mice and from rhythmic LL mice were similar (23.0 h versus 23.2 h, $P > 0.05$), as were their amplitudes and their standard deviations in period (±0.5 h versus ±0.5 h, $n = 735$ versus 783 neurons). Thus, constant light primarily affects phase organization among SCN neurons, not their properties as individual circadian oscillators.

Some of the mice that remained rhythmic in LL showed 'split' activity rhythms (**Fig. 3a**). In SCN from behaviorally split mice ($n = 5$), individual SCN neuronal rhythms were in synchrony within each SCN nucleus, but the cells in the left and right SCN oscillated in approximate antiphase, peaking about 12 h apart, similar to the activity bouts (**Fig. 3b–d**). Notably, each nucleus within the paired SCN structure showed coherent 24-h rhythmicity, and individual cell rhythms were never 'split', showing only a single peak of *Per1*-driven GFP fluorescence during each 24-h interval. The standard deviation of cell peak times in individual SCN nuclei from LL 'split' animals was 3.2 ± 1.5 h ($n = 10$ nuclei), similar to that of LL rhythmic animals and significantly less than that of LL arrhythmic animals ($P < 0.01$), again indicating a greater phase coherence within the individual nuclei of 'split' rhythmic mice compared to nuclei of arrhythmic mice. The calculated free running period of cellular rhythms in SCN from 'split' animals was also similar to that of the arrhythmic and rhythmic groups (22.7 ± 0.6 h, $P > 0.05$), as was the overall proportion of rhythmic neurons (936/1,033 neurons, 91%). These data demonstrating ongoing 'split' *in vitro* rhythms from SCN of animals that showed 'split' locomotor activity rhythms indicate that our imaging protocol faithfully preserves behaviorally relevant aspects of SCN function during the transition from *in vivo* to *in vitro* conditions. In addition, they reveal that the basis for the 'split' circadian organization is intercellular antiphasic synchrony between neurons of the paired SCN nuclei, as has been inferred from static gene expression assays of 'split' hamster SCN[4], rather than intra-SCN or intracellular splitting. The differences in neuronal synchrony in SCN from arrhythmic, rhythmic and split mice are illustrated in time-lapse videos (see **Supplementary Videos 1–3** online and high-resolution videos at http://vvrc.vanderbilt.edu/NatNeurosci2005OhtaYamazakiMcMahon).

Our findings resolve a basic question regarding the mammalian brain biological clock: at what level of organization is rhythmicity disrupted by external stimuli? Clearly, constant light disrupts circadian behavioral rhythms by disrupting the cellular organization of the SCN clock. The asynchronous but robust individual cellular rhythms in the SCN from arrhythmic LL mice indicate that disruption of behavioral and SCN tissue-level rhythmicity[5–7] is not the result of stopping the core molecular clock mechanism of individual neuronal oscillators. Circadian rhythm generation by mammalian biological clock neurons apparently persists at the cellular and molecular levels even as behavioral rhythmicity is blunted or reorganized by constant light; however, normal temporal organization of cellular rhythms within the clock nuclei is lost under these conditions. Coherent organization of neuronal population rhythms within the SCN is critical for driving robust circadian locomotor rhythms, and each nucleus of the SCN pair is evidently capable of independently driving a component of locomotor behavior. Loss of cellular synchrony is also a mechanism for damping circadian molecular oscillations in peripheral tissue circadian oscillators. At least some peripheral circadian oscillators can show self-sustained individual cell rhythms but lack coupling mechanisms that maintain tissue-level temporal organization (for example, fibroblast cell rhythms[8,9]). Thus, the SCN is distinguished from peripheral tissue oscillators by its ability to sustain phase coherence among its constituent neuronal oscillators through strong coupling interactions under normal circumstances. This ability is critical for the role of the

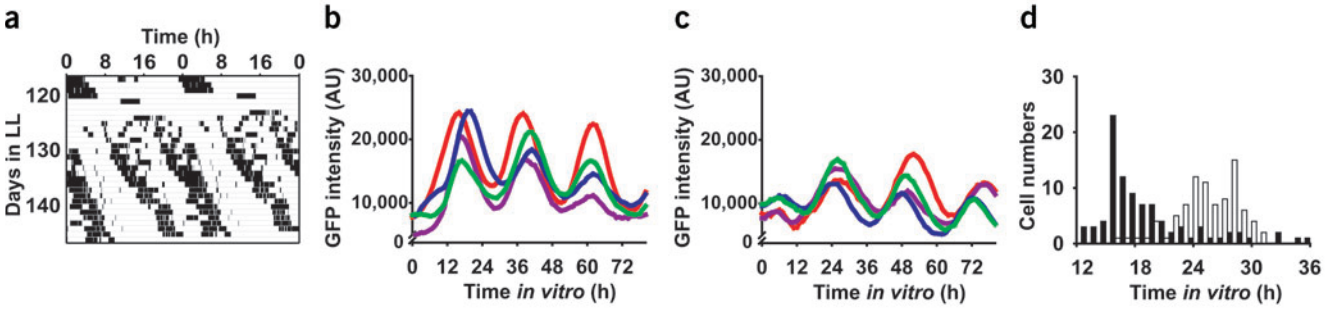

Figure 3 Behavioral and SCN rhythms from a 'split' rhythmic constant light–treated mouse. (**a**) Actogram of wheel running activity from a mouse that showed split rhythms in LL. (**b**,**c**) Individual SCN neuronal *Per1*:*GFP* rhythms in the left (**b**) and right (**c**) SCN. (**d**) Peak time histograms of individual neuronal rhythms. $n = 204$ cells.

SCN as a master pacemaker for orchestrating normal behavioral and physiological rhythmicity. The human circadian system can be subject to internal desynchronization in constant-light environments[10] that might be experienced in hospital intensive care units, during shift work or during prolonged space travel, as on planned Mars missions. Our results show the potential for mammalian biological clocks to remain functional during exposure to such environments and for chronotherapy to focus on issues of synchronization rather than rhythm generation.

Note: Supplementary information is available on the Nature Neuroscience website.

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COMPETING INTERESTS STATEMENT

The authors declare that they have no competing financial interests.

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