Constant Light Disrupts the Developing Mouse Biological Clock

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ABSTRACT: The central biological clock of the brain, contained within the suprachiasmatic nuclei (SCN) of mammals, orchestrates an orderly "internal day" of physiology and behavior. The developing biological clock begins to respond to light at an early stage and a particular concern in humans is whether light exposure has disruptive effects on the developing biological clock of infants exposed to constant lighting conditions in neonatal intensive care units (NICUs). Worldwide, eighteen million, or 14%, of newborns estimated to be of low birth weight, are exposed to artificial lighting environments in hospital nurseries annually. Here, we have tested whether constant light (LL) exposure disrupts the developing biological clock of mice, using a circadian reporter transgenic mouse model in which the organization of the central biological clock can be assayed by real-time gene expression imaging. We now find that LL has both acute and long-term disruptive effects on developing biological clocks and that cyclic lighting conditions are critical for developing circadian clocks to coordinate their molecular circadian mechanisms. This suggests that, from the perspective of developing circadian organization in humans, cyclic light conditions in NICUs are likely to be most appropriate for infants. (*Pediatr Res* **60:** 304–308, 2006)

The central biological clock of the brain, contained within the suprachiasmatic nuclei (SCN) of mammals, orchestrates an orderly "internal day" of physiology and behavior through its extensive influence on multiple organ systems, including the brain, heart, liver and lung (1). In many organisms, including humans, the biological clock and its control of circadian organization can be disrupted by exposure to constant light (LL) environments (2–6). Although previous studies have focused on effects of LL on adult organisms, in humans, a particular concern is whether LL has acute or long-term disruptive effects on the developing biological clock of preterm infants, who may be exposed to LL conditions in neonatal intensive care units (NICUs).

Clinical studies have suggested effects of light on preterm infants including disturbances of biologic rhythms and sleep states (7,8), effects on postnatal weight gain (7,9,10), and on visual development (11,12), indicating that the developing biological clock begins to respond to light at an early stage. However, there is no clear-cut scientific consensus on what constitutes an "appropriate" lighting environment for these infants and many NICUs are brightly and/or continuously lit.

Although there is a trend of late to use lower levels of illumination and to introduce cyclic lighting conditions, current recommendations for illumination in NICUs state that "Appropriate general lighting levels for NICUs have not been established." (11,13).

In this study we have tested whether exposure to LL disrupts the developing biological clock of mice, using a circadian reporter transgenic mouse model in which the cellular and molecular organization of the central biological clock can be assayed by real-time gene expression imaging and correlated with individual animal behavior (14–17). Our findings indicate that exposure of developing mice to LL acutely disrupts circadian behaviors as well as cellular synchrony within the central biological clock and has lasting effects on circadian organization that result in increased vulnerability to disruption by LL. Coherent circadian organization can be reestablished by switching to cyclic light conditions. These studies suggest that, from the perspective of developing circadian organization in humans, cyclic light conditions are likely to be most appropriate for preterm infants.

METHODS

Transgenic mice. Vanderbilt University bred B6C3H mice homozygous for the *mPeriod1* (*Per1*):d2EGFP transgene (17) were cross-bred to produce mice homozygous for the reporter transgene (*Per1*:GFP mice). Pregnant homozygous *Per1*:GFP mice (*n* = 10) were maintained under 12-h light/12-h dark cycles (LD, lights on at 06:00h). The day of delivery was designated as postnatal day 0 (P0). Each dam fed 5 pups from P0 through P20, the day of weaning. On P0 half of the group (5 dams with 25 pups) remained in the same LD conditions while the other half was transferred into constant light conditions (LL, 350 lux). On P20 the *Per1*:GFP signals in the SCN of pups from each condition were measured in *in vitro* sliced tissue culture. Animal care and use was reviewed and approved by the Vanderbilt University IACUC.

SCN sliced tissue culture. Mouse pups were humanely killed by cervical translocation and decapitation between 17:00 and 18:00 h (local time). Their brains were rapidly removed and placed in cold HBSS (14060-057; Invitrogen, Grand Island). Coronal sections (200-μm thick) including both left and right nuclei were made with a vibratome. SCN were cultured using the same methods as Ohta *et al.* (17).

Per1:GFP imaging. Cultures were maintained at 36°C in darkness, and their GFP signals were monitored for 3.5 d with a laser-scanning confocal microscope (Carl Zeiss, Jena, Germany). Three optical Z-plane sections of 54 μm that covered a total depth of 94 μm were acquired at hourly intervals. For analysis, the three image planes were combined and SCN or individual cells within SCN were delimited by a region of interest tool, and their fluorescence

Abbreviations: E, embryonic day; **LD**, 12-hr light/12-hr dark cycles; **LL,** constant light; **P,** postnatal day; ***Per1, Period 1*; SCN,** the suprachiasmatic nuclei

intensity was tracked over time with IP Lab software (Scanalytics, Fairfax, VA). Background fluorescence, assayed at each time-point from regions outside the borders of the SCN, was subtracted from SCN and cell values. Results are expressed as mean pixel intensity in arbitrary units (AU) within SCN and cells to normalize for SCN and cell size differences and plotted using a moving five-point average smoothing function. Cells were classified as non-rhythmic by the criteria previously used in Ohta *et al.* (17).

Analysis of single cell rhythms. The peak time distributions and standard deviations of *Per1*:GFP rhythms from single SCN neurons for LD and LL pups were calculated on the circadian cycle that occurred during hours 12–36 *in vitro* (Fig. 1*D,H*). Statistical comparisons for these data from the two experimental groups were performed by unpaired Student's *t*-tests ($p < 0.05$).
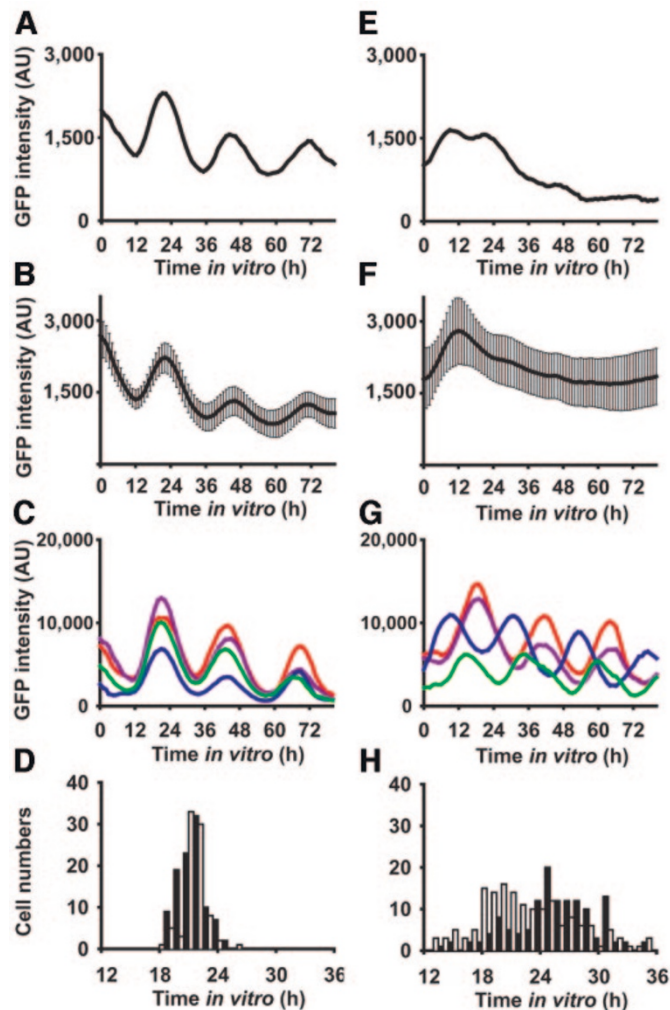
Activity rhythms. Wheel-running activity was recorded and analyzed using ClockLab software (Actimetrics, Chicago, IL). χ^2 periodogram analysis (18) of 14-d segments of wheel running activity was used to determine behavioral rhythmicity. Animals were judged to lack circadian behavioral organization when their Qp value for a 14-d segment of activity was below $p = 0.05$ or when they exhibited multiple period peaks that exceeded the $p = 0.05$ value

Figure 1. SCN Rhythms from an LD-Treated (*A–D*) and Constant Light-Treated (*E–H*) Mouse Pups. (*A*) Time-lapse SCN *Per1*:GFP fluorescence signals for 3.5 d *in vitro* from a mouse pup raised in LD. (*B*) Group data of *Per1*:GFP rhythms from 5 LD mouse SCN. (*C*) Individual SCN neuronal *Per1*:GFP rhythms from SCN in (*A*). Four representative cells (colored lines) are plotted for clarity. (*D*) Peak time histograms of individual neuronal rhythms in (*A*). Peak times of neurons in right SCN are plotted in black bars whereas those in left SCN are plotted in open bars. Histograms for this and the following figures are for hours 12–36 *in vitro* ($n = 191$ cells). (*E*) Time-lapse SCN *Per1*:GFP fluorescence signals from a mouse pup raised in LL. (*F*) Group data of *Per1*:GFP rhythms from 5 LL mouse SCN. (*G*) individual SCN neuronal *Per1*:GFP rhythms from SCN in (*E*). (*H*) Peak time histograms of individual neuronal rhythms from SCN in (*E*) $n = 307$ cells.

but were not circadian in period (*i.e.*, split rhythms). On P20, five pups were separated from each dam and transferred from their original cage into individual cages for their behavioral recordings. Two groups were prepared to evaluate the effects of the lighting conditions during the first three weeks of age on the development of their behavioral rhythmicity after weaning: in the first group, on P20 LL, pups were transferred into LD conditions (LL-LD pups); in the second group, after P20 LL, pups remained in LL (LL-LL pups). The same type of behavioral observation with mice was previously reported in (19).

RESULTS

Effects of constant light on the developing biological clock. To test the effect of constant light on the SCN during early development, we used *Per1*:GFP transgenic mice in which rhythmic activation of the *Per1* clock gene promoter can be monitored at the single cell level, in real-time, using time-lapse confocal microscopy (16,17). Pregnant dams were maintained on LD 12:12 light cycles during gestation and then, following parturition, dams and pups were exposed to either normal light-dark (LD) cycles or LL for the first three weeks of postnatal life while the locomotor activity rhythms of dams were assayed by wheel running. On P20 the SCN of LD and LL pups were dissected, explanted into organotypic culture and their *Per1* promoter-driven GFP fluorescence rhythms were recorded during the following 3.5 d *in vitro*. Individual SCN from LD pups exhibited clear circadian rhythms in gene expression averaging 3.04-fold in amplitude on the first circadian cycle *in vitro* ($\pm$ 0.66 mean $\pm$ SEM, throughout; 5 mice, Fig. 1*A*), similar in amplitude to SCN rhythms from LD-entrained adult mice (17). In addition, ongoing circadian rhythmicity was evident in the average SCN fluorescence data from all 5 preparations (Fig. 1*B*). We also tracked the molecular circadian rhythms of individual neurons within the SCN of LD pups and found that they also exhibited robust *Per1*:GFP rhythms and a high degree of synchrony (Fig. 1*C*). Peak time histograms of individual cell rhythms from LD pup SCN showed the majority of cell rhythms were synchronized in a single peak with a mean SD of 1.9 $\pm$ 0.1 h (Fig. 1*D*). Similar results were observed for individual cells in SCN of all LD pups ($n = 1,135$ cells, 10 nuclei, 5 mice).

In contrast, *Per1*:GFP circadian rhythms in SCN from LL pups were severely disrupted (Fig. 1*E*). The weak oscillations we observed in SCN from LL pups exhibited trough to peak amplitudes of 1.38-fold on average (*i.e.*, peaks 38% greater than troughs, $\pm$ 0.15, 5 mice). Group data demonstrated a transient increase in *Per1*:GFP during the first 12 h *in vitro*, perhaps due to the transition to *in vitro* conditions, but no evidence of sustained molecular rhythms in the population of SCN from LL pups, whether the data were aligned by local clock time (Fig. 1*F*), or by the time of peak expression in the first 24 h *in vitro* (not shown). In contrast to the whole SCN data, imaging of individual cellular rhythms within SCN from LL pups revealed the persistence of robust neuronal circadian rhythms in *Per1* promoter activation which were 1.5- to 4-fold in amplitude (Fig. 1*G*). The SCN neuronal rhythms of LL pups were, however, desynchronized in phase (Fig. 1*G*) and the peak times of individual neuronal rhythms from LL pups were widely dispersed (Fig. 1*H*). The SD of cell peak times for

SCN nuclei from LL pups was significantly greater than that for cells in SCN of LD pups (6.3 ± 0.8 h, $n = 10$ nuclei, $p < 0.01$). Similar robust, but desynchronized, neuronal rhythms were observed in the SCN of all LL pups assayed ($n = 1{,}113$ cells, 10 nuclei, 5 mice). The differences in neuronal synchrony in SCN from LD and LL pups are illustrated in time-lapse videos (see supplementary Videos 1 and 2 at: http://www.cas.vanderbilt.edu/ PediatrRes2005OhtaMitchellMcMahon).

Dams in both LD and LL conditions during their lactation periods exhibited circadian behaviors with LD dams exhibiting rhythms with periods near 24 h (24.0 ± 0.0 h, 5 mice, Fig. 2*A,B*) and LL dams showing slightly longer circadian periods (25.1 ± 0.8 h, 5 mice, Fig. 2*C,D*; $p < 0.05$). χ^2 periodogram analysis revealed all dams had significant circadian periods ($p < 0.05$) and that there were no significant differences in the amplitude of locomotor rhythms in LL and LD dams ($p > 0.05$).

Effects of constant light on activity rhythms at later developmental stages. To gain insight into potential long-term effects of exposing the developing biological clock to constant light, P20 LL pups were subsequently exposed to LL for four weeks while their locomotor activity rhythms were monitored by wheel running. Interestingly, 67% (26/39) of the LL-reared pups subsequently exposed to LL developed disrupted circadian locomotor activity patterns such as arhythmicity or multiple non-circadian rhythmic components (*i.e.*, "splitting"; Fig. 3*A–C*) within the first seven weeks of life. In contrast, in a separate study in which P20 LD-reared pups were subsequently exposed to LL, all pups exhibited coherent circadian rhythms for several weeks and only after 3–5 mo in LL did approximately 10% of these mice finally develop disrupted circadian locomotor activity patterns (17). The LD cycle is a strong synchronizer known to reset the biological clock (1). To rescue the disrupted circadian behavioral phenotypes observed in LL-reared pups, LD cycles were applied to LL pups after P20. All these pups exhibited strong, coherent circadian locomotor rhythms synchronized to the LD cycle from their first day in LD (Fig. 3*D–F*).
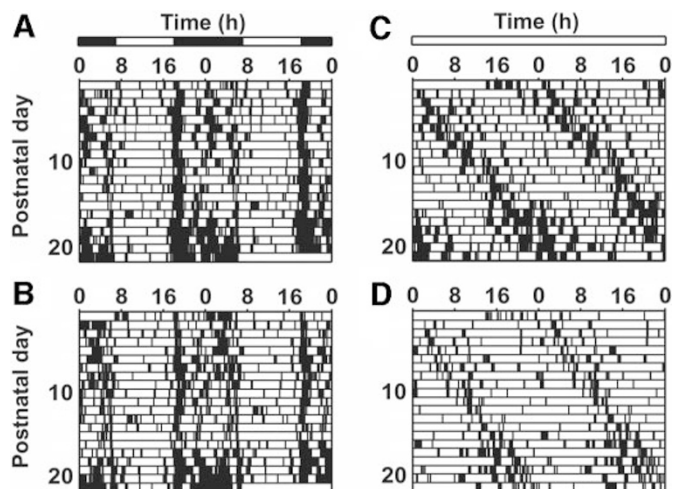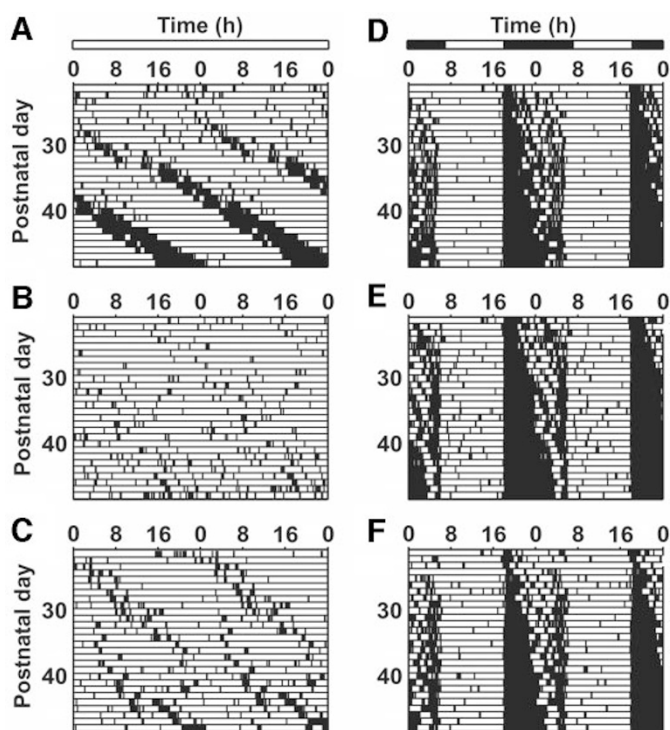
Figure 3. Behavioral rhythms from young mice raised continuously in constant light after birth (*A–C*) and young mice raised in constant light for the first three weeks after birth and then transferred to LD on postnatal day 20 (*D–F*). (*A*) Actogram of wheel running activity with circadian rhythmicity from an LL-treated pup. (*B, C*) Actograms of wheel running activity with disrupted circadian rhythmicity from LL-treated pups. (*D–F*) Actograms showing circadian activity of LD-treated pups.

DISCUSSION

Sensitivity of developing biological clocks to constant light. Our results provide compelling evidence that constant light disrupts developing biological clocks and overall circadian organization by desynchronizing the circadian rhythms of individual clock neurons within the SCN, but not by compromising the ability of single neurons to generate circadian rhythms. Cellular desynchronization is also the means by which constant light disrupts adult circadian clocks (17), but our data emphasize that developing biological clocks are more vulnerable to disruption by constant light and that cellular desynchronization during clock maturation can have long-lasting effects. Finally, our results show that light-dark cycles can protect developing circadian clocks from disruption by irregular lighting conditions.

The disruption of cellular organization of the neonatal SCN by LL was reflected in disrupted circadian behaviors in weanling mice, demonstrating that lighting conditions have significant influence on neonatal biological clocks and their ability to maintain internal temporal order. Continuous 7-wk exposure to LL from birth induced 67% of developing mice to have disrupted circadian locomotor activities, whereas all mice which had been exposed to LD cycles for the first three weeks of life maintained coherent circadian behaviors for at least 3 mo in LL and only 10% subsequently developed circadian disruptions with 3–5 mo of LL exposure (17). This indicates a critical and lasting effect of constant light *versus* cyclic light

Figure 2. Behavioral rhythms from dams during their lactation periods. (*A, B*) Actograms of wheel running activity from dam in LD. (*C, D*) Actograms of wheel running activity from dams in LL. The open and solid bars on the top of the actograms represent light and dark periods, respectively.

on developing clocks for the first three weeks of life in mice. Whereas there are many previous studies regarding maternal-fetal circadian interactions and its mechanisms (20–23) our studies concentrated on neonatal circadian development, holding lighting conditions during gestation constant, and then varying them during the neonatal period. Maternal-neonatal entrainment, in which pups synchronize to their mothers' circadian rhythms, has also been documented, but its physiologic mechanisms remain to be elucidated (24–27). Interestingly, our data demonstrates that even in the presence of maternal rhythmicity, light input can overcome maternal influence on pups' circadian rhythms, strongly influencing and even disrupting the maturing biological clock. Recently, vasoactive intestinal polypeptide (VIP) and gap junctions have been suggested to be key cellular coupling factors maintaining cellular synchrony within the SCN (28–30), and LL has been shown to suppress SCN VIP content in adult rodents (31). Developmental studies assessing the effects of LL on these modes of SCN neural communication may reveal the underlying mechanisms by which LL disrupts SCN maturation.

The neonatal period of SCN maturation in rodents is marked by the appearance of molecular clock gene rhythms as well as initiation of light responsiveness. In mice, the circadian rhythm of *Per1* mRNA expression starts at embryonic day 17 (E17), 2–4 d before birth (32), whereas in the rat, data indicate that *Per1* mRNA rhythms also begin between E20 and postnatal day 3 (P3) (33–36). The response of the SCN to light-dark cycles is already present at birth, as measured using c-fos gene induction (37–39). Recent studies have confirmed that the light entrainment pathway, consisting of intrinsically photoreceptive melanopsin-expressing retinal ganglion cells, is present and functional at P0 in both mice and rats (40,41). Whereas the molecular development of the human biological clock and melanopsin systems is less well defined, human infants do show both a developmental time course in their circadian organization and evidence of circadian responses to light, suggesting the possibility of similar underlying mechanisms (42,43).

Light-dark cycles as an effective chronotherapy for disrupted developing biological clocks. Our results show that constant light disrupts immature biological clocks and leads to abnormal circadian behaviors, however, once disrupted, coherent circadian behaviors could be restored by exposure to light-dark cycles. Exposure of the developing human circadian system to constant light also results in irregular sleep-wake patterns (7), reduced weight gain (7,9) and various neurologic complications (44). These types of constant-light environments might be experienced in hospital intensive care units or in lighting conditions at home in our night-active modern society. Our data suggest that in these situations restoration of appropriate light-dark cycles can be a strong and simple chronotherapy to protect developing circadian clocks from the effects of irregular lighting conditions.

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