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CRY1^{-/-} CIRCADIAN RHYTHMICITY DEPENDS ON SCN INTERCELLULAR COUPLING

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Abstract

In mammals, the suprachiasmatic nucleus (SCN) is the central pacemaker organizing circadian rhythms of behavior and physiology. At the cellular level, the mammalian clock consists of autoregulatory feedback loops involving a set of “clock genes”, including the *Cryptochrome* (*Cry*) genes, *Cry1* and *Cry2*. Experimental evidence suggests that *Cry1* and *Cry2* play distinct roles in circadian clock function. In mice, *Cry1* is required for sustained circadian rhythms in dissociated SCN neurons or fibroblasts, but not in organotypic SCN slices or at the behavioral level, whereas *Cry2* is not required at any of these levels. It has been argued that coupling among SCN cellular oscillators compensates for clock gene defects to preserve oscillatory function. Here we test this hypothesis in *Cry1*^{-/-} mice by first disrupting intercellular coupling *in vivo* using constant light (resulting in behavioral arrhythmicity) and then examining circadian clock gene expression in SCN slices at the single cell level. In this manner, we were able to test the role of intercellular coupling without drugs and while preserving tissue organization, avoiding the confounding influences of more invasive manipulations. *Cry1*^{-/-} mice (as well as control *Cry2*^{-/-} mice) bearing the PER2::LUC knockin reporter were transferred from a standard light:dark cycle to constant bright light (~650 lux) to induce arrhythmic locomotor patterns. In SCN slices from these animals, we used bioluminescence imaging to monitor PER2::LUC expression in single cells. We show that SCN slices from rhythmic *Cry1*^{-/-} and *Cry2*^{-/-} mice had similarly high percentages of functional single-cell oscillators. In contrast, SCN slices from arrhythmic *Cry1*^{-/-} mice had significantly fewer rhythmic cells than SCN slices from arrhythmic *Cry2*^{-/-} mice. Thus, constant light *in vivo* disrupted intercellular SCN coupling to reveal a cell-autonomous circadian defect in *Cry1*^{-/-} cells that is normally compensated by intercellular coupling *in vivo*.

Keywords

circadian; cryptochrome; constant light; intercellular communication; suprachiasmatic; arrhythmia

Introduction

The mammalian circadian system is a collection of cellular clocks located throughout the body that are synchronized by a primary pacemaker within the suprachiasmatic nucleus

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(SCN) (Dibner et al. 2010; Mohawk et al. 2012; Welsh et al. 2010). Non-SCN cells are autonomous oscillators, but in the absence of the SCN may desynchronize from one another (Nagoshi et al. 2004; Welsh et al. 2004), as they appear to lack local coupling within tissues (Stratmann and Schibler 2006). In contrast, the neuronal network within the SCN couples its component single-cell oscillators to produce a coherent circadian oscillation at the tissue level (Aton and Herzog 2005; Welsh et al. 2010). Unlike the independent oscillations of dissociated SCN neurons, which exhibit various periods and phases, SCN neurons within a slice adopt identical circadian periods and relatively stable phase relationships (Herzog et al. 2000; Welsh et al. 1995). Such coordinated rhythms can be measured longitudinally in SCN slices *in vitro* by single-cell bioluminescence imaging of clock gene expression using *Per1-luc* or *PER2::LUC* reporters (Evans et al. 2011; Foley et al. 2011; Yamaguchi et al. 2003; Yan et al. 2007). The precise relative phasing of individual cells is preserved from cycle to cycle, in a complex pattern of distinct phases and amplitudes, suggesting that SCN coupling is mediated by specific neural circuits, rather than by homogeneous neuronal interactions. The mechanism of this coupling involves neuronal firing and chemical synapses, with vasoactive intestinal polypeptide (VIP) playing a particularly important role (Aton and Herzog 2005; Welsh et al. 2010).

Within individual cells, circadian oscillations are driven by a delayed negative feedback loop in which CLOCK/BMAL1 heterodimers activate transcription of *Period (Per)* and *Cryptochrome (Cry)* genes. After transcriptional and post-transcriptional delays, the PERs and CRYs form complexes that inhibit CLOCK/BMAL1 activity, thus inhibiting their own transcription (Mackey 2007; Takahashi et al. 2008). In time, proteasomal degradation of PERs and CRYs relieves the inhibition and allows the cycle to begin anew. The roles of putative molecular clock components have traditionally been evaluated by testing behavioral rhythms of knockout animals. Both *Cry1*^{-/-} and *Cry2*^{-/-} mice have intact rhythms of locomotor activity in constant darkness, with shorter period in *Cry1*^{-/-} mice and longer period in *Cry2*^{-/-} mice relative to wild type (van der Horst et al. 1999; Vitaterna et al. 1999). At the level of single cells and non-SCN tissues, however, *Cry1*^{-/-} produces a more profound defect (Liu et al. 2007). Whereas non-SCN tissues, fibroblasts, and dissociated SCN neurons cultured from *Cry2*^{-/-} mice are self-sufficient oscillators, only rare, transient rhythms are observed in single cells and non-SCN tissues from *Cry1*^{-/-} mice. In contrast, SCN slices from both *Cry1*^{-/-} and *Cry2*^{-/-} mice are strongly rhythmic with short and long period, respectively, in parallel with behavior. Thus, some property of the SCN *in vivo*, which is preserved in slices but not in dispersed cell culture, compensates for the *Cry1*^{-/-} genetic defect to produce strong rhythms. Since an important distinction between an SCN slice and dispersed SCN neurons and non-SCN tissues is the presence of coupling in the former, this suggests that *Cry1*^{-/-} cells may require intercellular communication across the network to generate sustained rhythms, and that SCN coupling rescues oscillatory function in the face of this genetic defect.

Here we test the hypothesis that coupling among SCN single cell oscillators, rather than some other aspect of tissue organization, is responsible for the compensatory effect observed in *Cry1*^{-/-} SCN slices. We reasoned that if SCN coupling is required to compensate for a *Cry1*^{-/-} defect, then disrupting coupling in *Cry1*^{-/-} SCN slices should impair rhythmicity. Although SCN coupling can be disrupted by genetic or pharmacological manipulations targeting cellular coupling mechanisms (e.g., VIP gene knockout, or toxins to block synaptic transmission), such manipulations can also adversely affect oscillatory function of individual SCN neurons (Aton et al. 2005; Deery et al. 2009). We wished to avoid such confounding effects on cellular circadian function by disrupting coupling in a less invasive manner.

Specifically, SCN coupling can be altered *in vivo* by manipulation of environmental lighting conditions, such as exposure to constant bright light (LL). In nocturnal rodents, LL

lengthens the free-running period and decreases the duration and level of locomotor activity (Aschoff 1960). Further, some rodents held under LL develop arrhythmic activity patterns that reflect desynchrony among SCN neurons (Ohta et al. 2005). Importantly, LL disrupts coupling without otherwise altering the characteristics of single cell rhythms (Ohta et al. 2005). Therefore, to test whether SCN coupling is required to compensate for *Cry1* deficiency, we disrupted SCN coupling in *Cry1*^{-/-} or *Cry2*^{-/-} mice through *in vivo* exposure to LL, and then tested *in vitro* for defects in PER2::LUC rhythms displayed by SCN neurons within slices collected from these mice. We predicted that if *Cry1*^{-/-} rhythmicity depends on SCN coupling, then disrupting this coupling should impair single-cell rhythmicity in *Cry1*^{-/-} slices, but not in *Cry2*^{-/-} slices.

Materials and Methods

Animals

Homozygous *Cry1*^{-/-} and *Cry2*^{-/-} mice (van der Horst et al. 1999) were crossed with homozygous PER2::LUC knock-in mice (Yoo et al. 2004) to generate homozygous reporter knockout mice as in (Liu et al. 2007). Prior to use in experiments, mice were group-housed without running wheels and entrained to a 14h light: 10h dark cycle (LD 14:10, lights on: 0600 PST). Ambient temperature was maintained at 22 ± 2°C, with food (Purina Rodent Chow #5001, St. Louis, MO) and water available *ad libitum*. At 7–9 wks of age, male *Cry1*^{-/-} and *Cry2*^{-/-} mice were transferred to individual wheel-running cages located within light-tight chambers and exposed to constant bright light (LL, 650 lux at cage level). All animal studies were conducted in accordance with the regulations of the Committee on Animal Care and Use at the University of California, San Diego.

Behavioral Analyses

Wheel-running activity rhythms were analyzed visually to identify animals that had become arrhythmic and remained arrhythmic for at least two weeks prior to dissection. Locomotor activity patterns were submitted to χ^2 periodogram analyses (ClockLab, Actimetrics), and animals were verified to be arrhythmic when the χ^2 periodogram failed to detect a significant period in the circadian range (18–30h, $p < 0.05$, 1-tailed). Animals that maintained coherent circadian rhythms under LL were selected as rhythmic controls for each genotype. For animals with coherent locomotor rhythms, circadian time (CT) of dissection was calculated by use of a linear regression fit to activity onsets (defined as CT12) displayed over the preceding 6–7 days.

Explant Culture

Animals were humanely killed by decapitation. For all mice, SCN dissection was performed at a standard clock time (1000 PST). SCN slices (200 μ m) were cut in the coronal plane with a motorized vibratome. Each slice was trimmed near the edges of the SCN and cultured on a membrane (Millicell-CM, PICMORG030-50, Millipore) in a sealed dish containing 1.2 mL HEPES-buffered serum-free explant medium (Dulbecco's Modified Eagle's Medium, DMEM, GIBCO 12100-046) supplemented with 1.2 g/L NaHCO₃, 10 mM HEPES, 4 mM glutamine, 25 U/ml penicillin, 25 μ g/ml streptomycin, 2% B-27 (GIBCO 17504-044), and 1 mM luciferin (BioSynth, L-8220).

Bioluminescence imaging

To study rhythmic circadian clock gene expression in SCN slices at single cell resolution, PER2::LUC bioluminescence imaging was performed for 3–5 days, with 30 min temporal resolution (Welsh et al. 2005). Immediately after dissection, SCN slices were transferred to the stage of an inverted microscope (Olympus IX71) housed within a dark room and

maintained at 36°C within a heated lucite chamber (Solent Scientific, UK). Images were collected with an Olympus 10x UPlanSApo objective and transmitted to a CCD camera (Spectral Instruments SI800, Tucson, AZ) cooled to -90°C. To assess long-term changes in bioluminescence rhythmicity, a subset of SCN slices from arrhythmic *Cry1*^{-/-} mice were transferred from the camera to a LumiCycle luminometer (Actimetrics, Inc.) kept inside a standard tissue culture incubator at 36°C. Bioluminescence from each dish was continuously recorded with a photomultiplier tube (PMT) for 70 sec every 10 minutes. Slices were retained within the luminometer for at least four weeks, and the medium was changed once a week.

Data Analysis

Bioluminescence images were analyzed with MetaMorph software (Molecular Devices). Spurious events were removed by using the minimum value in pixel-wise comparisons of two consecutive images. Images were corrected for bias and dark current by background subtraction. For single cell analyses, selected cells were those that were easily discriminated from adjacent cells and continued to exhibit bioluminescence over the entire 3–4 day imaging experiment. Luminescence intensity for each cell was measured within a manually defined region of interest that was adjusted to accommodate cell movement. Data were processed further using Excel (Microsoft), where luminescence values were converted to photons/min based on rated quantum efficiency and gain of the camera. Luminescence time series were then imported into LumiCycle Analysis software (Actimetrics, Inc.). The first 12 h of each time series was excluded due to high transient luminescence upon dissection. A linear baseline was subtracted from the raw data (polynomial order = 1), and the baseline-subtracted data were fit by a sine wave from which period and phase were determined. Single cells were defined as rhythmic using a best-fit sin wave with criteria of a goodness-of-fit (percent of total variance accounted for by the fitted curve) ≥ 25%, a circadian period (18–36 hrs), and amplitude ≥ 15 (Liu et al. 2007). Phases were plotted and analyzed with Oriana software (Kovach Computing, UK). All other statistical tests were conducted with JMP software (SAS Institute, Cary, NC). Data in figures are means ± SEM.

Results

Cry1^{-/-} and *Cry2*^{-/-} mice were both highly susceptible to constant bright light, such that a great majority of animals of each genotype (87% of *Cry1*^{-/-}, 70% of *Cry2*^{-/-}) developed arrhythmic locomotor patterns within seven weeks after transfer to LL (Figure 1). *Cry1*^{-/-} and *Cry2*^{-/-} mice did not differ significantly in the proportion that developed arrhythmic behavioral activity patterns during the first seven weeks in LL (Figure 1, Fisher's Exact Test $\chi^2(1) = 1.04$, $p = 0.36$). Of animals that became arrhythmic, genotype did not significantly influence the rate at which arrhythmia developed (Figure 1, $t(23) = -0.39$, $p = 0.69$).

Regardless of behavioral phenotype, all SCN slices displayed PER2::LUC circadian rhythms *in vitro* (Figure 2). For rhythmic mice of each genotype, PER2::LUC rhythm phases were clustered according to *in vivo* circadian phase (Figure 2A; Rayleigh test, $p < 0.01$) rather than by dissection time (Figure 2B; Rayleigh test, $p > 0.05$). SCN slices from rhythmic *Cry1*^{-/-} and *Cry2*^{-/-} mice did not differ in peak time ($t(6) = 1.8$, $p = 0.12$) or rhythm amplitude ($t(5) = -2.1$, $p = 0.09$). In contrast, for arrhythmic mice of each genotype, PER2::LUC rhythm phases were clustered according to dissection time (Figure 2C; Rayleigh test, $p < 0.01$). Relative to SCN slices from arrhythmic *Cry2*^{-/-} mice, SCN slices from arrhythmic *Cry1*^{-/-} mice peaked 9h earlier ($t(9) = 20.4$, $p < 0.0001$) and displayed lower amplitude rhythms ($t(9) = -2.4$, $p = 0.04$).

As a test of the correspondence between *in vivo* and *in vitro* rhythms, we next analyzed the period of PER2::LUC rhythms displayed by SCN slices and neurons. Circadian period

displayed by SCN slices and single cells reflected the previously described effects of *Cry* genotype, even in slices from mice rendered behaviorally arrhythmic (Table 1). Overall, *Cry1*^{-/-} SCN slices had significantly shorter periods than *Cry2*^{-/-} SCN slices (Genotype: $F(1,14) = 114.4$, $p < 0.0001$), and this difference was accentuated in SCN slices from arrhythmic mice (Genotype X Phenotype: $F(1,14) = 8.9$, $p = 0.0098$). Of single cells displaying a period in the circadian range (18–36h), *Cry1*^{-/-} cells had a significantly shorter period than *Cry2*^{-/-} cells (Genotype: $F(1,14) = 53.9$, $p < 0.0001$), regardless of behavioral phenotype (Genotype X Phenotype: $F(1,14) = 2.3$, $p = 0.15$).

To assess synchrony among cells, we analyzed PER2::LUC rhythms of single neurons in SCN slices from rhythmic and arrhythmic *Cry2*^{-/-} mice (Figure 3A). Relative to slices from rhythmic mice, slices from arrhythmic mice tended to have more variance in phase among cells, less concentration of phase, and a shorter vector radius for the cells ($\chi^2(1) = 3.33$, $p < 0.07$ for each test). Although not statistically significant at the $p = 0.05$ criterion level for individual tests, the consistency of the trend across all three measures indicates that coupling was compromised by LL, though not completely abolished.

Cry genotype and LL-induced behavioral phenotype of an animal interacted to influence the prevalence and robustness of rhythms displayed by neurons within an SCN slice (Figure 3A–B, Figure S1–4, $F(1,14) = 4.6$, $p < 0.05$). SCN slices from rhythmic *Cry1*^{-/-} and *Cry2*^{-/-} mice displayed a high percentage of rhythmic neurons (88% and 93%, respectively). SCN slices from arrhythmic *Cry2*^{-/-} mice had a similarly high percentage of rhythmic neurons (89%). In contrast, SCN slices from arrhythmic *Cry1*^{-/-} mice had a much lower percentage of rhythmic neurons (45%, Tukey's HSD $p < 0.01$). For rhythmic cells, *Cry* genotype and behavioral phenotype also affected the robustness of rhythms as measured by “goodness-of-fit”, or percentage of variance accounted for by a fitted sine wave (Figure 3C). *Cry1*^{-/-} cells displayed a lower goodness-of-fit than *Cry2*^{-/-} cells ($F(1,14) = 8.88$, $p < 0.01$), and goodness-of-fit tended to be lower in cells from arrhythmic mice than in those from rhythmic mice ($F(1,14) = 3.94$, $p = 0.067$). SCN cells from arrhythmic *Cry1*^{-/-} mice had the least robust rhythms compared to SCN cells from each of the three other groups (Figure 3C, Tukey's HSD $p < 0.01$).

SCN slices from arrhythmic *Cry1*^{-/-} animals were viable, even though they had fewer rhythmic cells. Average brightness of PER2::LUC bioluminescence requires ATP and is one indicator of tissue viability. As indicated by PER2::LUC expression averaged over the first two days *in vitro*, SCN slices from *Cry1*^{-/-} mice were at least as bright as those from *Cry2*^{-/-} mice (Figure 4A), and much brighter in the case of rhythmic *Cry1*^{-/-} mice (Genotype: $F(1,14) = 7.5$, $p = 0.016$; Phenotype: $F(1,14) = 6.1$, $p = 0.027$; Genotype X Phenotype: $F(1,14) = 6.94$, $p = 0.0196$). Also, SCN slices from arrhythmic *Cry1*^{-/-} animals eventually recovered robust PER2::LUC rhythms after a few medium changes (Figure 4B).

Discussion

SCN neurons deficient in *Cry1* exhibit prominent and persistent circadian rhythms of PER2 expression in SCN slice preparations but not in dispersed culture (Liu et al. 2007). We hypothesized that this is because sustained circadian rhythmicity in *Cry1*^{-/-} cells requires intercellular coupling, rather than some other property of the intact SCN slice that is missing in dispersed cultures. In the present study, we test this hypothesis by disrupting SCN coupling *in vivo* through exposure to LL, and then assessing oscillatory function of individual cells in SCN slices by PER2::LUC imaging. LL can suppress locomotor activity rhythms in *Cry1*^{-/-} and *Cry2*^{-/-} mice, as in wild type mice (Spoelstra and Daan 2008). In wild type mice, LL-induced behavioral arrhythmia is due to desynchronization among SCN neurons (Ohta et al. 2005), and we hypothesized that the same would be true for *Cry*

knockout mice. If so, then we reasoned that *Cry2*^{-/-} neurons, which (like wild type neurons) are competent single cell oscillators in dispersed culture, should exhibit strong but desynchronized rhythms in SCN slices taken from arrhythmic animals. We further reasoned that disrupting SCN intercellular coupling through *in vivo* exposure to LL should cripple the oscillatory function of *Cry1*^{-/-} but not *Cry2*^{-/-} neurons, mimicking the results of cell dispersal in culture. We therefore predicted that LL would (1) produce behavioral arrhythmicity of *Cry* knockout mice, (2) preserve rhythmicity but reduce synchrony of SCN neurons in slices from arrhythmic *Cry2*^{-/-} mice and (3) reduce rhythmicity of SCN neurons in slices from arrhythmic *Cry1*^{-/-} mice. The present results are consistent with all three predictions and support the hypothesis that *Cry1*^{-/-} cells are not cell-autonomous oscillators but require SCN coupling to maintain rhythmic expression of PER2.

We considered the possibility that *Cry1*^{-/-} and *Cry2*^{-/-} mice might respond differently to LL than wild type mice, because previous studies found altered responses to light in both genotypes: decreased induction of *Per1* in SCN (Thresher et al. 1998; Vitaterna et al. 1999), but also larger phase delays of behavioral rhythms (Spoelstra and Daan 2008; Thresher et al. 1998). In the only previous study examining effects of LL in *Cry* knockout mice (Spoelstra and Daan 2008), locomotor activity rhythms were suppressed similarly in *Cry1*^{-/-}, *Cry2*^{-/-}, and wild type mice, but incidence of complete arrhythmia was not reported, and LL intensity and duration were limited. In the present study, we found that bright LL suppressed locomotor activity rhythms rapidly in both *Cry1*^{-/-} and *Cry2*^{-/-} mice: 87% of *Cry1*^{-/-} mice and 70% of *Cry2*^{-/-} mice became arrhythmic within seven weeks in LL. In previous work, wild type mice in LL have generally developed arrhythmia more slowly (Ohta et al. 2005; Pickard 1994; Sollars et al. 2002; Spoelstra and Daan 2008; Steinlechner et al. 2002; Vitaterna et al. 1993), but methodological differences (e.g., mouse strain, LL intensity and duration) limit direct comparisons with the present results. While it is uncertain whether LL is more disruptive for the behavior of *Cry*-deficient mice than that of wild type mice, it is clear that *Cry1*^{-/-} and *Cry2*^{-/-} mice rapidly become arrhythmic in bright LL.

Based on previous work using the *Per1-GFP* mouse (Ohta et al. 2005), we predicted that LL-induced behavioral arrhythmicity would reflect desynchrony among SCN neurons. At the single cell level, PER2::LUC rhythm phases of single cells were indeed more broadly distributed in *Cry2*^{-/-} SCN slices from arrhythmic mice than in *Cry2*^{-/-} slices from rhythmic mice. However, even in SCN slices from behaviorally arrhythmic mice, cell phases were not randomly distributed. This suggests that our LL manipulation was sufficient to render mice behaviorally arrhythmic but did not completely abolish synchrony within SCN slices. Consistent with this, all SCN slices, even those from arrhythmic mice, had clearly discernible circadian PER2::LUC rhythms at the level of the whole slice. Since other studies have observed arrhythmicity in SCN slices from arrhythmic rodents exposed to long-term LL (Granados-Fuentes et al. 2004; Ohta et al. 2005), it may be that more time under LL is required to completely desynchronize SCN neurons. Another possibility is that desynchronized SCN rhythms in behaviorally arrhythmic mice are more susceptible to the resetting effects of dissection, which could partially restore synchronization *in vitro*. Consistent with this possibility, and as also found in a previous study of *Per1-luc* rats in LL (Yoshikawa et al. 2005), SCN slice phases of arrhythmic animals were determined by time of dissection, unlike the SCN slices of rhythmic animals, whose phases were clustered according to *in vivo* circadian time. We also found a nearly antiphasic relationship between slices from arrhythmic *Cry1*^{-/-} and *Cry2*^{-/-} mice, suggesting genotypic differences in phase-shifting effects of dissection, possibly related to the altered photic resetting responses previously reported for *Cry1*^{-/-} and *Cry2*^{-/-} mice (Spoelstra and Daan 2008; Thresher et al. 1998; Vitaterna et al. 1999). It is also of interest that differences in circadian period of SCN slices and cells from *Cry1*^{-/-} and *Cry2*^{-/-} mice were consistent with the period phenotypes described previously (Liu et al. 2007; van der Horst et al. 1999; Vitaterna et al. 1999).

Finally, SCN slices from arrhythmic *Cry1*^{-/-} mice showed reduced amplitude relative to SCN slices from arrhythmic *Cry2*^{-/-} mice, which reveals a persistent influence of LL that is consistent with a reduced amplitude of individual cells and/or greater desynchrony among cells within *Cry1*^{-/-} slices. In summary, both single cell and whole SCN slice rhythm phenotypes *in vitro* are consistent with partial rather than complete desynchrony of cells by LL, with some genotype-specific effects on phase and period.

Despite the lack of complete desynchrony, LL-induced arrhythmia was sufficient to produce a genotypically specific reduction in the rhythmicity of *Cry1*^{-/-} neurons in SCN slices. Wild type mice were not included in this study, but *Cry2*^{-/-} mice provided an excellent control group since *Cry1*^{-/-} and *Cry2*^{-/-} mice both lack a core clock gene and display rapid arrhythmia under LL, but nevertheless differ fundamentally in the effects of LL on SCN neuron rhythmicity. The proportion of individual cells showing detectable PER2::LUC rhythms was reduced in SCN slices from behaviorally arrhythmic *Cry1*^{-/-} mice, relative to arrhythmic *Cry2*^{-/-} mice, rhythmic *Cry2*^{-/-} mice, and rhythmic *Cry1*^{-/-} mice. We also examined another measure of rhythmicity: the goodness-of-fit to a best fit sine wave in the circadian period range. This goodness-of-fit was lower in *Cry1*^{-/-} than in *Cry2*^{-/-} cells, tended to be lower in cells from arrhythmic mice than in cells from rhythmic mice, and was lowest of all in *Cry1*^{-/-} cells from arrhythmic mice. Thus, even though the LL manipulation used here did not appear to completely desynchronize SCN cells, partial disruption of SCN intercellular coupling was sufficient to reduce rhythmicity of *Cry1*^{-/-} cells in SCN slices.

This reduced rhythmicity observed in cells of SCN slices from arrhythmic *Cry1*^{-/-} mice does not appear to be related to reduced tissue viability. SCN slices from arrhythmic *Cry1*^{-/-} mice did have lower amplitude PER2::LUC rhythms. However, if *Cry1* deletion compromised cell viability, we would expect to see a similarly reduced amplitude in *Cry1*^{-/-} slices from rhythmic mice, as well as a reduction in the average brightness of PER2::LUC bioluminescence in *Cry1*^{-/-} slices. On the contrary, rhythms of *Cry1*^{-/-} and *Cry2*^{-/-} slices from rhythmic mice were of comparable amplitude, and *Cry1*^{-/-} slices were either just as bright as *Cry2*^{-/-} slices (for arrhythmic mice) or substantially brighter than *Cry2*^{-/-} slices (for rhythmic mice). The greater average PER2::LUC brightness in *Cry1*^{-/-} SCN slices from rhythmic mice was also observed previously for individual dispersed *Cry1*^{-/-} cells (Liu et al. 2007), likely reflecting the strong repressor function of CRY1 protein at the *Per2* gene promoter. The viability of SCN slices from arrhythmic *Cry1*^{-/-} mice was also demonstrated by the recovery of robust rhythmicity observed after several medium changes.

Completely abolishing SCN intercellular coupling by mechanical dispersal of cells results in markedly reduced rhythmicity of *Cry1*^{-/-} cells (Liu et al. 2007). In the present study, treating mice with LL was designed to disrupt SCN intercellular coupling without the concomitant destruction of other tissue properties of the SCN slice, thereby testing whether reduced rhythmicity is specifically due to disruption of circadian coupling. With the relatively gentle manipulation of LL exposure, coupling was only partially disrupted, so it is not surprising that the proportion of rhythmic *Cry1*^{-/-} cells was not as low as in dispersed cells, where coupling is abolished altogether. Nevertheless, reduced rhythmicity of *Cry1*^{-/-} SCN neurons by LL-induced disruption of intercellular coupling supports the hypothesis that *Cry1*^{-/-} cells do indeed depend on such coupling to maintain robust circadian function.

It remains unknown what underlying mechanisms explain different CRY1 and CRY2 contributions to single cell oscillatory function, i.e. why uncoupled *Cry1*^{-/-} cells are nearly arrhythmic, whereas *Cry2*^{-/-} cells are strongly rhythmic with long period. Based on the behavior of *Cry* mutant mice in constant darkness, it was originally proposed that *Cry1* and *Cry2* serve redundant roles in maintaining the molecular oscillator but antagonistic roles in determining its pace (van der Horst et al. 1999; Vitaterna et al. 1999). While effects on

period length are highly reproducible, other data suggest that *Cry1* and *Cry2* deletions do not have identical effects on rhythm strength and precision even at the behavioral level (van der Horst et al. 1999; Oster et al. 2002; Spoelstra and Daan 2008; Thresher et al. 1998). At the molecular level, both CRY1 and CRY2 are potent repressors of CLOCK:BMAL1 transcription that can induce nuclear translocation of PER (Griffin et al. 1999; Kume et al. 1999; Lee et al. 2001). CRY1 and CRY2 display an overall 70–80% similarity in amino acid sequence (Hirayama et al. 2003; Ozturk et al. 2007; Sancar 2004; Tamanini et al. 2007), but the C-terminal tail domain is generally less conserved (Chaves et al. 2006; Hirayama et al. 2003). CRY1 and CRY2 may differ in transcriptional and/or posttranslational modifications that produce different affinities for PER or other clock components. CRY1 and CRY2 may also have different levels of protein expression and/or amplitude of rhythms (Okamura et al. 1999; Vitaterna et al. 1999; Yagita et al. 2002). Finally, a recent study demonstrated that the precise phase of rhythmic *Cry1* expression is essential to its function, whereas *Cry2* expression need not be rhythmic for normal circadian function (Ukai-Tadenuma et al. 2011). Further studies are needed to delineate the specific mechanisms underlying the distinct roles of CRY1 and CRY2 in the intracellular oscillator.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

Effects of LL on locomotor behavior of *Cry*-deficient mice. A. Representative double plotted wheel-running actograms of *Cry1*^{-/-} and *Cry2*^{-/-} mice that were transferred to LL on the first day of the record. The majority of *Cry*-deficient mice quickly became arrhythmic after transfer to LL, but a minority of animals retained circadian rhythms for the first few weeks under LL. B. Proportion of *Cry1*^{-/-} and *Cry2*^{-/-} animals that developed arrhythmic activity patterns within seven weeks under LL (left) and latency to develop arrhythmia (right). Numbers within columns represent total sample sizes for animals held under LL for at least seven weeks and used for behavioral analyses. R: rhythmic mice, AR: arrhythmic mice.

Figure 2.

Effects of genotype and LL-induced behavioral arrhythmicity on PER2::LUC rhythms of SCN slices *in vitro* illustrated by baseline-subtracted time series. A–B. PER2::LUC rhythms of SCN slices from rhythmic *Cry1*^{-/-} (n = 5) and *Cry2*^{-/-} (n = 3) mice were significantly clustered according to circadian time (A) but not dissection time (B). Rayleigh plots depict the phase distribution of peak PER2::LUC on the first cycle *in vitro*, plotted in angular degrees. Lines represent mean angular vectors and those extending outside the inner circle indicate significant clustering for that group as determined by the Rayleigh test. C. PER2::LUC rhythms of SCN slices from arrhythmic *Cry1*^{-/-} (n = 7) and *Cry2*^{-/-} (n = 5) mice were clustered according to dissection time and genotype.

Figure 3. Reduced PER2::LUC rhythmicity of single cells in SCN slices from behaviorally arrhythmic *Cry1*^{-/-} mice. A. Representative background-subtracted time series of PER2::LUC rhythms displayed by SCN neurons from rhythmic and arrhythmic *Cry1*^{-/-} and *Cry2*^{-/-} mice (five neurons per panel illustrated with different colors). More examples are shown in Figures S1–S4. B. SCN slices from arrhythmic *Cry1*^{-/-} mice had fewer rhythmic neurons relative to slices from rhythmic *Cry1*^{-/-} mice, arrhythmic *Cry2*^{-/-} mice, or rhythmic *Cry2*^{-/-} mice. C. The goodness-of-fit (percentage of total variance in the PER2::LUC trace accounted for by a best fit sine wave) was reduced in SCN cells from *Cry1*^{-/-} mice, and was lowest in *Cry1*^{-/-} cells from arrhythmic mice. Average number of cells extracted per slice: 40 cells. * $p < 0.05$. * Tukey's HSD, $p < 0.05$.

Figure 4.

Viability of *Cry1*^{-/-} SCN slices from arrhythmic mice. A. SCN slices from *Cry1*^{-/-} animals were at least as bright as *Cry2*^{-/-} slices (right). * Different from all other groups, Tukey's HSD, $p < 0.05$. B. Example of a SCN slice from an arrhythmic *Cry1*^{-/-} mouse that initially displayed very low amplitude rhythms, but then high amplitude and sustained rhythms after two medium changes.

Table 1Period of PER2::LUC rhythms reflects *Cry* genotype

	SCN Slices		SCN Neurons	
	Rhythmic	Arrhythmic	Rhythmic	Arrhythmic
<i>Cry1</i> ^{-/-}	23.02 ± 0.35	22.01 ± 0.14	22.28 ± 0.35	24.81 ± 0.42
<i>Cry2</i> ^{-/-}	25.89 ± 0.87	27.10 ± 0.28	28.54 ± 0.29	28.53 ± 0.23
<i>p</i> value	< 0.05	< 0.0001	< 0.0001	< 0.0001

* Note: Rhythmic and arrhythmic indicate behavioral state prior to dissection