



Published in final edited form as:

*Heart Rhythm*. 2015 June ; 12(6): 1306–1314. doi:10.1016/j.hrthm.2015.02.019.

## The Cardiomyocyte Molecular Clock Regulates the Circadian Expression of *Kcnh2* and Contributes to Ventricular Repolarization

Elizabeth A. Schroder, Ph.D.<sup>a</sup>, Don E. Burgess, Ph.D.<sup>a</sup>, Xiping Zhang, Ph.D.<sup>a</sup>, Mellani Lefta, Ph.D., M.D.<sup>a</sup>, Jennifer L. Smith, B.S.<sup>a</sup>, Abhijit Patwardhan, Ph.D.<sup>b</sup>, Daniel C. Bartos, Ph.D.<sup>a</sup>, Claude S. Elayi, M.D.<sup>c</sup>, Karyn A. Esser, Ph.D.<sup>a</sup>, and Brian P. Delisle, Ph.D.<sup>a</sup>

<sup>a</sup>Center for Muscle Biology, Department of Physiology, University of Kentucky, Lexington, KY

<sup>b</sup>Center for Biomedical Engineering, University of Kentucky, Lexington, KY

<sup>c</sup>Department of Cardiology, University of Kentucky, Lexington, KY

### Abstract

**Background**—Sudden Cardiac Death (SCD) follows a diurnal variation. Data suggest the timing of SCD is influenced by circadian (~24 hour) changes in neurohumoral and cardiomyocyte-specific regulation of the heart's electrical properties.

**Objective**—The basic helix-loop-helix transcription factors BMAL1 and CLOCK coordinate the circadian expression of select genes. We tested whether *Bmal1* expression in cardiomyocytes contributes to K<sup>+</sup> channel expression and diurnal changes in ventricular repolarization.

**Methods**—We utilized transgenic mice that allow for the inducible cardiomyocyte-specific deletion of *Bmal1* (iCS *Bmal1*<sup>-/-</sup>). We used quantitative PCR, voltage-clamping, promoter-reporter bioluminescence assays, and electrocardiographic (ECG) telemetry.

**Results**—Although several K<sup>+</sup> channel gene transcripts were downregulated in iCS *Bmal1*<sup>-/-</sup> mouse hearts, only *Kcnh2* exhibited a robust circadian pattern of expression that was disrupted in iCS *Bmal1*<sup>-/-</sup> hearts. *Kcnh2* underlies the rapidly activating delayed-rectifier K<sup>+</sup> current (*I<sub>Kr</sub>*), and *I<sub>Kr</sub>* recorded from iCS *Bmal1*<sup>-/-</sup> ventricular cardiomyocytes was ~50% compared to control myocytes. Promoter-reporter assays demonstrated that the human *Kcnh2* promoter is transactivated by the co-expression of BMAL1 and CLOCK. ECG analysis showed iCS *Bmal1*<sup>-/-</sup> mice developed a prolongation in the heart rate corrected QT (QTc) interval during the light (resting)-phase. This was secondary to an augmented circadian rhythm in the uncorrected QT interval without a corresponding change in the RR interval.

---

Corresponding Author: Brian P. Delisle, Department of Physiology, University of Kentucky College of Medicine, 800 Rose St. MS508, Lexington, KY 40536, Telephone: (859) 323-2797, brian.delisle@uky.edu.

**Conflicts of Interests:** Brian Delisle has a research contract with Gilead Scientific.

**Publisher's Disclaimer:** This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

**Conclusion**—The molecular clock in the heart regulates the circadian expression of *Kcnh2*, modifies K<sup>+</sup> channel gene expression and is important for normal ventricular repolarization. Disruption of the cardiomyocyte circadian clock mechanism likely unmasks diurnal changes in ventricular repolarization that could contribute to an increased risk of cardiac arrhythmias/SCD.

### Keywords

circadian rhythm; repolarization; ECG; QTc; *Bmal1*; *Kcnh2*

---

## INTRODUCTION

The discovery that the incidence of sudden cardiac death (SCD) follows a time-of-day dependence suggests that circadian factors participate in the initiation of lethal arrhythmias.<sup>1, 2</sup> Biological circadian rhythms are evolutionarily conserved cycles that repeat every ~24 hours, and synchronize behavior and physiology with the daily environment.<sup>3, 4</sup> The cardiovascular system shows robust circadian rhythms in blood pressure, heart rate, electrocardiographic (ECG) properties, and gene expression.<sup>5</sup> Historically, the early morning rise in SCD has been linked to the circadian variations in myocardial ischemic events and autonomic signaling, however, more recent studies also support a cardiomyocyte-specific circadian or molecular clock mechanism in the heart.<sup>6-8</sup>

The cellular mechanism responsible for generating biological circadian rhythms is a conserved gene regulatory network composed of a transcriptional-translational feedback loop called the molecular clock, which is expressed in almost all cells including cardiomyocytes.<sup>9, 10</sup> The positive limb of the molecular clock is formed by the transcription factors BMAL1 (brain muscle arnt-like1) and CLOCK (circadian locomotor output control kaput), and the negative limb is regulated by PERs (Period 1, 2, and 3) and CRYs (Cryptochrome 1 and 2). Circadian changes in gene expression and cardiac function are linked to the cardiomyocyte molecular clock mechanism.<sup>11, 12</sup> Bray and colleagues (2008) showed transgenic mice that selectively overexpress a dominant-negative CLOCK mutation in cardiomyocytes alters gene expression in both atrial and ventricular myocytes, heart rate, contractile function, and metabolism.<sup>13</sup> We recently found that the inducible cardiomyocyte-specific deletion of *Bmal1* (iCS *Bmal1*<sup>-/-</sup>) in adult mice disrupts molecular clock signaling in the heart, causes a loss in the circadian expression of the Na<sup>+</sup> channel gene *Scn5a* (Nav1.5), decreases macroscopic Na<sup>+</sup> current ( $I_{Na}$ ) recorded from isolated ventricular cardiomyocytes, slows the heart rate, and increases the frequency of cardiac arrhythmias.<sup>8</sup> Emerging evidence suggests that the cardiomyocyte molecular clock might regulate the expression of several cardiac K<sup>+</sup> channels and ventricular repolarization as well.<sup>6, 7</sup> However, these studies do not distinguish the relative contribution of the cardiomyocyte molecular clock from circadian clock signaling in other tissues. The purpose of this study is to determine how *Bmal1* expression in cardiomyocytes contributes to changes in K<sup>+</sup> channel expression and ventricular repolarization.

## METHODS

### Inducible deletion of *Bmal1* in adult cardiomyocytes

All animal procedures were conducted in compliance with the guidelines of the Association for Assessment and Accreditation of Laboratory Animal Care and were approved by the Institutional Animal Care and Use Committee at University of Kentucky. The inducible cardiac specific *Bmal1* (iCS *Bmal1*) mouse model used for these studies was described previously.<sup>8</sup> *Cre*-recombination was activated once the mice reached 12 weeks of age by intraperitoneal injections of tamoxifen (2 mg/day) for 5 consecutive days. This injection protocol causes effective recombination without any obvious long-term tamoxifen toxicity. Mice were housed in a 12-hour light/dark (L/D) cycle for all experiments except the time course collection, which is detailed below.

### Circadian Collections

Circadian collections were done as described previously.<sup>8</sup> Briefly, 64 iCS *Bmal1* mice (mixed gender, 14–16 weeks of age) were housed in individual cages in light boxes and entrained to a 12-hour L/D cycle for 2 weeks. Mice had ad libitum access to food and water. Following the entrainment period, half of the mice were injected with vehicle and the other half with tamoxifen, generating 32 control iCS *Bmal1*<sup>+/+</sup> and 32 iCS *Bmal1*<sup>-/-</sup> mice, respectively. Two-weeks after the final injection, the mice were then released into constant darkness (D/D), and after 30 hours in D/D, we collected the ventricular apex every 4 hours from 3–4 animals in each group for a total of 8 time points. Circadian collections from control WKY (Wistar Kyoto) rats were done similarly. RNA was prepared for quantitative PCR (qtPCR) using TaqMan (Applied Biosystems) assays to examine the gene expression of *Kcnd2*, *Kcnh2*, *Kcnip2*, *Kcna5*, *Kcnb1*, *Kcnj2*, and *Kcnq1* mRNA. The  $\Delta\Delta$ CT method was used for the quantification of qtPCR data in the circadian collections. Gene expression in each sample was shown as the relative value compared with the mean vehicle value.

### Adult cardiomyocyte isolation and electrophysiology

Adult ventricular myocytes were isolated for voltage-clamp experiments as described previously.<sup>8</sup> Isolations were performed at 6–8 weeks following vehicle or tamoxifen injections. Voltage-clamp was performed with an Axopatch 200B patch-clamp amplifier (Axon Instruments, Foster City, CA) and pClamp10 software (Axon Instruments, Foster City, CA). Because  $I_{K_r}$  recorded using conventional approaches in mouse ventricular myocytes is small and contaminated by other currents, we isolated  $I_{K_r}$  using Cs<sup>+</sup> as the charge carrier similar to that described previously.<sup>14–16</sup> Unlike other K<sup>+</sup> channels,  $I_{K_r}$  channels readily permeate Cs<sup>+</sup> in the absence of K<sup>+</sup> and using Cs<sup>+</sup> as the charge carrier allows us to measure  $I_{K_r}$  directly (without current subtraction using  $I_{K_r}$  blockers, which improves the signal to noise ratio). The extracellular solution contained (in mM): NaCl 5, CsCl 90, CaCl 1, MgCl 1.2, glucose 11, TEA-Cl 10, HEPES 5 (pH 7.3 set with CsOH), and the pipette (intracellular) solution contained CsF 120, CsCl 20, EGTA 10, TEA-Cl 10, Na<sub>2</sub>ATP 1, HEPES 5 (pH 7.3 set with CsOH). Heterologously expressed *Kcnh2* channels (Kv11.1) in HEK293 cells generated large currents with similar gating properties as native  $I_{K_r}$  (data not shown). The holding potential was –140 mV. Cells were depolarized from –80 to 40 mV in 10 mV increments for 1 sec, followed by a test-pulse to –80 mV. The peak

current measured during the test-pulse to  $-80$  mV was plotted as a function of the pre-pulse potential and the individual current-voltage ( $I$ - $V$ ) relations were described using the following Boltzmann equation:

$$I = (I_{\text{MIN}} - I_{\text{MAX}}) / (1 + e^{[(V - V_{1/2})/k]}) + I_{\text{MAX}}$$

$I_{\text{MIN}}$  is the minimally activated current,  $I_{\text{MAX}}$  is the maximally activated current,  $V_{1/2}$  is the midpoint potential for half maximal current activation, and  $k$  is the slope factor of activation (mV/ $e$ -fold change). All voltage-clamp experiments from isolated iCS *Bmal1*<sup>+/+</sup> or iCS *Bmal1*<sup>-/-</sup> ventricular myocytes were performed at 22–23°C within 4 hours of isolation.

### Promoter-reporter bioluminescence assays

Heterologous expression of promoter-reporter constructs was performed in C2C12 myoblasts similar to that described previously.<sup>8</sup> For control studies, we utilized the *Per1* promoter-reporter construct 6.8Per1-Luc. We cloned the 734 bp human *Kcnh2* 5'-promoter sequence into the pGL3 basic vector (Promega) using human genomic DNA (hKcnh2-luc). The primers used for amplification of the 5' promoter sequence were 5'-CACGGTACC TCTTAGTCGCTAATCTGGGGTGG -3' (forward) and 5'-CACGCTAGC ACCGGCATCCTGAGCCCAT -3' (reverse). The sequence of the hKcnh2 promoter-reporter construct (hKcnh2-luc) was verified by DNA sequencing at the Advanced Genetic Testing Center, University of Kentucky. Lipofectamine2000 was used at a 3:1 ratio. To control for the total amount of in each transfection, transfected DNA was adjusted to 390 ng with the empty pcDNA3.1 plasmid. Forty-eight hours after transfection, luminescence of the lysate (20  $\mu$ l) was measured using the Dual-Luciferase Reporter Assay System (Promega) in a Lumat LB 9507 (EG&G Berthold). Similar to what we have shown previously in NIH/3T3 expression of the mouse *Bmal1* and *Clock* cDNA cloned in pcDNA3.1 (mBmal1- and mClock-pcDNA3.1, respectively) enhanced *Per1* promoter activity several fold in C2C12 cells (data not shown).<sup>8</sup> We assessed hKcnh2-luc promoter-reporter expression in four conditions: 1) promoter-reporter only, 2) co-transfected with mBmal1- and mClock-pcDNA3.1; 3) co-transfected with mClock-pcDNA3.1 and a missense mutation in *Bmal1* cDNA that changes an arginine to an alanine at amino acid 91 (mBmal1R91A-pcDNA3.1) and disrupts BMAL1 function; and 4) co-transfected with mBmal1- and a deletion mutant in *Clock* cDNA at amino acid 19 (mClock 19) that disrupts CLOCK function.<sup>17</sup> These latter two experiments with the mutant constructs served as negative controls. The pRL null vector was co-transfected in each experiment to provide a control for variations in transfection efficiency.

### ECG telemetry

In vivo ECG telemetry was performed as described previously.<sup>8, 18</sup> Briefly, mice were anesthetized with isoflurane and telemetry transmitter units (PhysioTel ETA-F10; Data Sciences International) were implanted in the peritoneal cavity. Two ECG leads were secured near the apex of the heart and the right acromion. Mice were allowed to recover for 2 weeks. We used two complementary methods to quantify the QT interval.<sup>18</sup> The first method was to manually measure and calculate the QTc interval using a modified version of

the Bazett's formula adjusted for mice and described in detail by Mitchell and colleagues (1998).<sup>19</sup> For the second approach, we used an automated program written in Matlab (Mathworks) to compute hourly uncorrected QT intervals similar as described previously.<sup>18</sup> Briefly, ECG traces recorded for each hour were aligned to the peak of the R wave to generate an hourly averaged trace and the QT calculation was done using the threshold method. The Q was defined as the base of the QRS complex (where the slope of the profile changed from negative or isoelectric to positive) and the T was defined as the point where the ECG returned 95% of the way from the T-wave minima to the isoelectric level. The hourly RR and QT intervals were plotted for ~ 2–3 consecutive days and were fit with the following non-linear sinusoidal model:

$$\text{Interval} = A * \cos(2\pi(t - \tau)/T) + m$$

This model allowed calculation of the period (T) - the time between the peak amplitudes; phase ( $\tau$ ) - time of the peak rhythm in reference to the onset of the lights on (Zeitgeber Time or ZT= 0); the circadian amplitude (A) - 1/2 of the peak-to-trough amplitude; and the circadian mean (m) - a rhythm-adjusted mean halfway between the peak and trough amplitude.

### Statistical analysis

Results are reported as mean  $\pm$  SEM where  $P < 0.05$  is considered significant. For gene expression studies, the statistical JTK\_CYCLE package was used to identify circadian rhythms in the qPCR expression profiles. JTK\_CYCLE reliably distinguishes between rhythmic and non-rhythmic transcripts and exhibits an increased resistance to outliers in the data, giving it considerably greater sensitivity and specificity.<sup>20</sup> Additionally, JTK\_CYCLE accurately measures the period, phase, and amplitude of cycling transcripts, facilitating comparative analyses. Unpaired t-tests were performed to determine significance for electrophysiological measures, and statistical analysis of the ECG telemetry data was done using a one-way ANOVA with the Bonferroni post-hoc analysis (Prism, GraphPad Software, Inc.).

## RESULTS

### Kcnh2 is a candidate clock controlled gene

We investigated the role of cardiomyocyte molecular clock in the regulation of cardiac  $K^+$  channel gene expression important in mouse and human ventricular repolarization (Supplemental Table 1). To identify  $K^+$  channel gene candidates that are expressed in a circadian pattern, we utilized the high resolution CircaDB microarray dataset for the heart (<http://circadb>), which analyzed gene expression every 2 hours for 48 hours in combination JTK\_CYCLE analysis.<sup>21</sup> We then confirmed these results in control (iCS *Bmal1*<sup>+/+</sup>) hearts by measuring the mRNA expression levels every 4 hours for 28 hours using qPCR and JTK\_CYCLE statistical analysis (Figure 1A). If an mRNA is determined to be circadian following JTK-CYCLE analysis in both datasets, then we concluded that the mRNA transcripts are circadian. The results of the two datasets suggest that only *Kcnd2* and *Kcnh2* transcripts exhibit a robust circadian oscillation in control hearts. We next tested whether

cardiac *Bmal1* expression contributed to the circadian expression pattern of *Kcnd2* and *Kcnh2* by determining whether the circadian oscillation in transcripts was lost in iCS *Bmal1*<sup>-/-</sup> hearts. The circadian pattern of the *Kcnh2* transcript, but not the *Kcnd2* transcript, was lost in iCS *Bmal1*<sup>-/-</sup> hearts (Figure 1A). This suggested that *Kcnh2* is controlled by the cardiomyocyte molecular clock but the central clock likely regulates the circadian expression of *Kcnd2*. Surprisingly, we found that the average 24-hour expression levels of several other cardiac K<sup>+</sup> channel transcripts (which did not follow a circadian pattern) were lower in iCS *Bmal1*<sup>-/-</sup> hearts (Figure 1B). These data suggested that the cardiomyocyte molecular clock signaling might indirectly contribute to the expression of non-circadian K<sup>+</sup> channel genes.

### Disruption of the cardiomyocyte molecular clock decreases I<sub>Kr</sub>

Since *Kcnh2* is a candidate clock controlled gene, we focused our mechanistic investigation on the consequences that deleting *Bmal1* has on the functional expression of *Kcnh2*. *Kcnh2* encodes the pore-forming  $\alpha$ -subunit that conducts the rapidly activating delayed-rectifier K<sup>+</sup> current (*I<sub>Kr</sub>*).<sup>22</sup> Loss-of-function mutations in *Kcnh2* are a common cause of congenital long QT syndrome (LQTS), and *I<sub>Kr</sub>* channels are prolific drug targets that contribute to drug-induced LQTS.<sup>23</sup> *I<sub>Kr</sub>* recorded from iCS *Bmal1*<sup>-/-</sup> ventricular myocytes hearts was ~50% smaller than control ventricular myocytes (Figure 2A). The reduction in *I<sub>Kr</sub>* was consistent with a decrease in gene expression, because there were no obvious differences in the gating properties of the *I<sub>Kr</sub>* (Figure 2B).

### The circadian pattern of *Kcnh2* expression is conserved and co-expression of BMAL1 and CLOCK transactivates the human *Kcnh2* promoter

We next determined whether *Kcnh2* transcripts follow a circadian pattern of expression in another species. We measured *Kcnh2* expression every 4 hours for 28 hours from rat ventricle, similar to what was done for the iCS *Bmal1* mice. JTK\_CYLCE analysis of the *Kcnh2* mRNA confirmed that the transcripts followed a robust circadian pattern (Figure 3A). Furthermore, we investigated whether heterologous expression of BMAL1 and CLOCK could enhance the transcriptional activity of the cloned human *Kcnh2* promoter. Heterologous expression of hKCNH2-Luc in C2C12 cells showed that, compared to cells expressing hKCNH2-Luc alone, co-expressing mBmal1 and mClock DNA increased luciferase activity several fold (Figure 3B). Importantly, negative control experiments that co-expressed cDNAs with mutations (Bmal1R91A or Clock<sup>-19</sup>) that disrupt BMAL1 or CLOCK function did not enhance hKCNH2-Luc luciferase activity (Figure 3B). Together the data suggest that the circadian expression of *Kcnh2* is conserved, and BMAL1 and CLOCK can enhance the transcriptional activity of the human *KCNH2* promoter.

### Disruption of the cardiomyocyte molecular clock unmask a circadian rhythm in ventricular repolarization

Since disrupting the cardiomyocyte molecular clock in the mouse heart decreased the expression of several K<sup>+</sup> channel transcripts important for mouse ventricular repolarization, we tested whether the QTc intervals measured from iCS *Bmal1*<sup>-/-</sup> mice were different compared to iCS *Bmal1*<sup>+/+</sup> mice. Similar to what was previously shown with in vivo ECG

telemetry of wild type mice, the QTc interval was constant in the iCS *Bmal1*<sup>+/+</sup> mice throughout the day.<sup>19</sup> However, the QTc intervals measured from the iCS *Bmal1*<sup>-/-</sup> showed a time-of-day prolongation during the light-phase (Figure 4). These data are surprising because they suggest that disruption of *Bmal1* in cardiomyocytes unmasked a diurnal prolongation in the QTc interval that is not normally seen in control mice.

In order to understand how the cardiomyocyte-specific deletion of *Bmal1* prolongs the QTc interval during the light-phase, we quantified the circadian rhythms in the hourly RR and uncorrected QT intervals for control and iCS *Bmal1*<sup>-/-</sup> mice over several days. The data were fit with a non-linear sinusoidal function to calculate the period, phase, circadian mean, and circadian amplitude of the rhythms (Figure 5; Table 1). The rhythms in the RR and QT intervals were all circadian (~24 hrs) and peaked 2–5 hours after the beginning of the light phase (ZT 2–5). The mean and amplitude of the circadian rhythm in the RR intervals were not different between the iCS *Bmal1*<sup>+/+</sup> and iCS *Bmal1*<sup>-/-</sup> mice, but the mean and the amplitude of the circadian rhythm in the QT intervals were larger for the iCS *Bmal1*<sup>-/-</sup> mice (Figure 5; Table 1). Importantly, these changes were only seen after inducing the deletion of *Bmal1* with tamoxifen treatment (Table 1), and they did not occur in parental Mer-Cre-Mer mice treated with tamoxifen (n=4–5 mice each, p>0.05, data not shown). Together, these data show the cardiomyocyte deletion of *Bmal1* increased the QTc interval during the light-phase because it augmented the circadian rhythm in the uncorrected QT interval without a corresponding change in the RR interval.

## DISCUSSION

The circadian molecular clock is a highly conserved, cell-autonomous, transcriptionally mediated mechanism that provides an evolutionary advantage by optimizing an organism's physiology to anticipate the daily variation in the environment.<sup>24</sup> To date there is only tangential and circumstantial evidence linking the cardiomyocyte molecular clock to circadian changes in K<sup>+</sup> channel expression and cardiac electrophysiology. In 2003, Yamashita and colleagues hypothesized that the expression of certain cardiac K<sup>+</sup> channel genes might be circadian, and they showed that *Kcnd2* and *Kcna5* transcripts followed a circadian pattern of expression in the female rat heart.<sup>6</sup> Jeyaraj and colleagues (2012) recently published that *Kcnd2* and *Kchip2* are also expressed in a circadian pattern in the heart.<sup>7</sup> However, neither study directly addressed the role that the cardiomyocyte molecular clock has on the circadian expression of these channels.

There are several similarities and differences between the results and conclusions from Yamashita and company (2003), Jeyaraj and colleagues (2012), and our study.<sup>6,7</sup> All three studies agree that *Kcnd2* transcripts are expressed in a circadian pattern and *Kcnj2* transcripts are not. However in contrast to Yamashita and colleagues, we conclude that *Kcnh2* transcripts are expressed in a circadian pattern but *Kcna5* transcripts are not. Moreover, unlike Jeyaraj and colleagues, we did not find that *Kchip2* transcripts follow a circadian pattern of expression. The reasons for these discrepancies likely include the statistical approaches used to determine which transcripts exhibit a circadian pattern of expression and the sampling frequency of the transcripts tested (typically every 3–5 hours over a single day). Yamashita and colleagues relied on an ANOVA around a single time

point whereas Jeyaraj and colleagues used consiner analysis. We used JTK\_CYCLE analysis, which is a statistical approach that is resistant to outliers in the data and results in considerably greater sensitivity and specificity.<sup>20</sup> The identification of circadian transcripts was done using data from a high resolution data set (CircaDB) in which expression is tracked every 2 hours for 48 hours coupled with our own JTK\_CYCLE analyses.<sup>21</sup> Importantly, we also found that *Kcnh2* transcripts undergo a robust circadian pattern of expression in rat hearts (Figure 3A). Together, these datasets allow us to confidently conclude that *Kcnh2* transcripts follow a circadian pattern of expression.

To directly test the role that the cardiomyocyte molecular clock has on the circadian expression pattern of *Kcnd2* and *Kcnh2* transcripts, we utilized a transgenic mouse model that disrupts the core circadian clock gene *Bmal1* in adult cardiomyocytes. We found that the circadian expression of *Kcnh2* (but not *Kcnd2*) transcripts was disrupted in iCS *Bmal1*<sup>-/-</sup> hearts (Figure 1A), the corresponding  $I_{Kr}$  was smaller in these iCS *Bmal1*<sup>-/-</sup> ventricular myocytes (Figure 2), and co-expressing *Bmal1* and *Clock* cDNA caused the transactivation of the cloned human *Kcnh2* promoter in C2C12 cells (Figures 3B). Together, these data strongly suggest that *Kcnh2* is a direct target of the cardiomyocyte molecular clock mechanism. Our finding that the circadian expression of *Kcnd2* is not altered in iCS *Bmal1*<sup>-/-</sup> hearts suggests its expression is likely influenced by clock mechanisms originating outside the heart (i.e. neurohumoral signaling). Consistent with this, germline *Bmal1*-null mice do not show a circadian pattern of *Kcnd2* expression in the heart.<sup>7</sup> Perhaps more surprising, is the finding that disruption of the molecular clock also caused the downregulation of a number of different K<sup>+</sup> channel transcripts not expressed in a circadian pattern (Figure 1B). These data argue that several downstream mechanisms related to cardiomyocyte molecular clock signaling (i.e. regulation of transcription factors, metabolism, etc) can also influence K<sup>+</sup> channel expression. Identification of the mechanism(s) which drives the circadian expression of *Kcnd2* and the downregulation of non-circadian K<sup>+</sup> channel transcripts warrants further investigation.

Surprisingly, we found that the deletion of *Bmal1* from cardiomyocytes unmasked a time-of-day prolongation in the QTc interval during the light-phase (Figure 4). This finding is counterintuitive because it suggests that the cardiomyocyte molecular clock functions to *limit* the circadian rhythm in the QT interval. These data are exciting because they highlight a new role for the molecular clock mechanism: not only does the molecular clock mechanism initiate anticipatory circadian rhythms to adjust to daily changes in the environment, but it can also help to offset or blunt circadian rhythms that might prove detrimental.

Based on the ECG data, our working model for how the cardiomyocyte molecular clock contributes to ventricular repolarization is quite different than Jeyaraj and colleagues.<sup>7</sup> Jeyaraj and colleagues suggested that the transcription factor, *Krüppel-like factor 15* gene (*Klf15*) is a direct target of BMAL1 in the heart, and they used several different transgenic *Klf15* mouse models to argue KLF15 underlies the circadian expression of *Kchip2* in the heart. They speculate that, since *Kchip2* is an obligatory subunit for the transient outward K<sup>+</sup> current ( $I_{to}$ ), the KLF15-mediated circadian expression of *Kchip2* produces a daily rise and fall in  $I_{to}$  to generate a circadian rhythm in the QTc interval of WT mice. We and several



other studies have not been able to resolve a circadian rhythm in the QTc interval in WT or control mice.<sup>18, 19</sup> More importantly, our work suggests that disruption of the cardiomyocyte molecular clock likely downregulates the expression for a number of different K<sup>+</sup> genes. A loss in overall K<sup>+</sup> channel expression would be expected to prolong the QT interval. Interestingly, our data suggest that disruption of the cardiomyocyte molecular clock contributes to a disproportionate prolongation of the QT interval during the light-phase when the heart rate is slowest in mice (Figure 5; Table 1). These findings are analogous to reports investigating the circadian relation between the RR interval and QT interval in a transgenic mouse model of long QT syndrome (LQTS), as well as clinical data investigating the relation between the RR and QT intervals in long QT syndrome patients.<sup>18, 25</sup>

There are several limitations to this study. Since this work is done in mice, the question as to whether a link exists between cardiac ion channel expression, diurnal alterations in ventricular repolarization, and susceptibility to lethal arrhythmias underlie the diurnal variation in SCD in humans requires further study. There are undoubtedly multiple factors that contribute to the heightened early morning susceptibility to SCD, including changes in autonomic signaling.

## CONCLUSION

This is the first work to clearly demonstrate that intrinsic circadian mediators, including the cardiomyocyte molecular clock, are modulators of the electrical properties in the heart, which possibly contributes to the daily variation in SCD.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

## Acknowledgments

### Acknowledgement of Financial Support: Funding Sources

This work was supported by the following NIH grants RC1ES018636 and AR55246 (KAE), and R01 HL087039 (BPD).

## Abbreviations

<b>SCD</b>	Sudden Cardiac Death
<b>ECG</b>	Electrocardiographic
<b>qtPCR</b>	quantitative PCR
<b>BMAL1</b>	Brain muscle arnt-like1
<b>Clock</b>	Circadian locomotor output control kaput
<b>PER</b>	Period
<b>CRY</b>	cryptochrome
<b>iCS <i>Bmal1</i></b>	inducible cardiomyocyte specific deletion of <i>Bmal1</i>

<b>ZT</b>	Zeitgeber time
<b>QTc</b>	heart rate corrected QT
$I_{Na}$	Na <sup>+</sup> current
$I_{Kr}$	rapidly activating delayed-rectifier K <sup>+</sup> current
<b>LQTS</b>	long QT syndrome
<b>Klf15</b>	krüppel-like factor 15

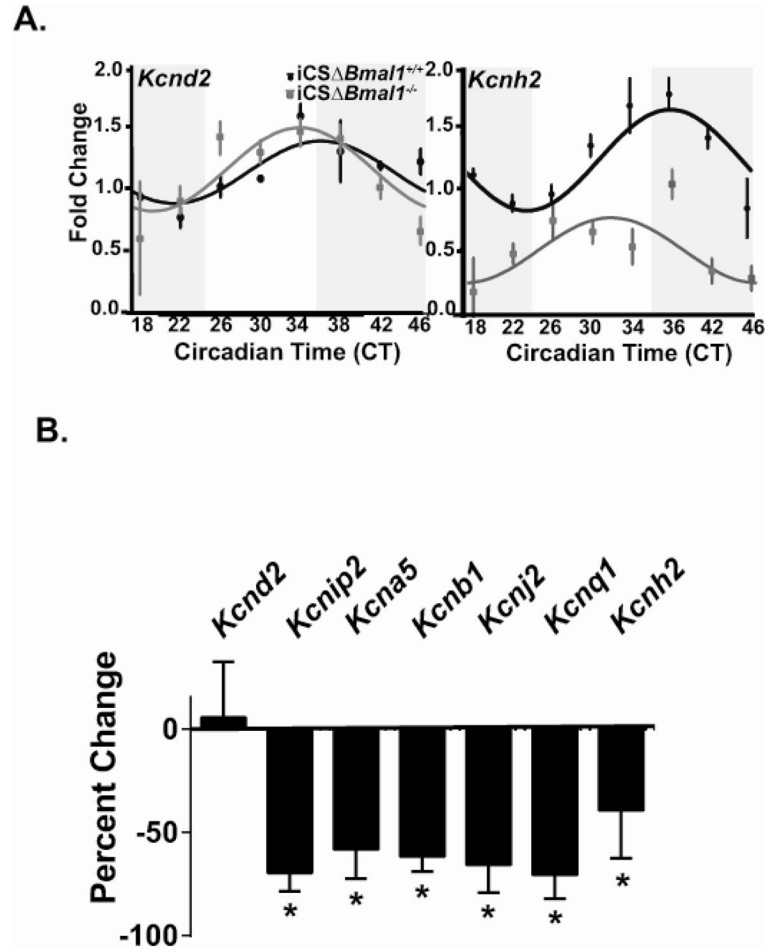
## Bibliography & References Cited

1. Muller JE, Ludmer PL, Willich SN, Tofler GH, Aylmer G, Klangos I, Stone PH. Circadian variation in the frequency of sudden cardiac death. *Circulation*. Jan.1987 75:131–138. [PubMed: 3791599]
2. Willich SN, Levy D, Rocco MB, Tofler GH, Stone PH, Muller JE. Circadian variation in the incidence of sudden cardiac death in the Framingham Heart Study population. *The American journal of cardiology*. Oct 1.1987 60:801–806.
3. Schibler U. The daily rhythms of genes, cells and organs. *Biological clocks and circadian timing in cells*. EMBO Rep. Jul.2005 6(S9-13)
4. Schibler U, Naef F. Cellular oscillators: rhythmic gene expression and metabolism. *Current opinion in cell biology*. Apr.2005 17:223–229. [PubMed: 15780601]
5. Martino TA, Sole MJ. Molecular Time: An Often Overlooked Dimension to Cardiovascular Disease. *Circulation Research*. Nov 20.2009 105:1047–1061. [PubMed: 19926881]
6. Yamashita T, Sekiguchi A, Iwasaki YK, Sagara K, Inuma H, Hatano S, Fu LT, Watanabe H. Circadian variation of cardiac K<sup>+</sup> channel gene expression. *Circulation*. Apr 15.2003 107:1917–1922. [PubMed: 12668525]
7. Jeyaraj D, Haldar SM, Wan X, et al. Circadian rhythms govern cardiac repolarization and arrhythmogenesis. *Nature*. Mar 1.2012 483:96–99. [PubMed: 22367544]
8. Schroder EA, Lefta M, Zhang X, Bartos DC, Feng HZ, Zhao Y, Patwardhan A, Jin JP, Esser KA, Delisle BP. The cardiomyocyte molecular clock, regulation of *Scn5a*, and arrhythmia susceptibility. *Am J Physiol Cell Physiol*. May 15.2013 304:C954–965. [PubMed: 23364267]
9. Mohawk JA, Green CB, Takahashi JS. Central and Peripheral Circadian Clocks in Mammals. *Annual Review of Neuroscience*. 2012; 35:445–462.
10. Young ME, Razeghi P, Taegtmeier H. Clock genes in the heart: characterization and attenuation with hypertrophy. *Circ Res*. Jun 8.2001 88:1142–1150. [PubMed: 11397780]
11. Durgan DJ, Hotze MA, Tomlin TM, Egbejimi O, Graveleau C, Abel ED, Shaw CA, Bray MS, Hardin PE, Young ME. The intrinsic circadian clock within the cardiomyocyte. *American journal of physiology Heart and circulatory physiology*. Oct.2005 289:H1530–1541. [PubMed: 15937094]
12. Young ME, Brewer RA, Pelicciari-Garcia RA, et al. Cardiomyocyte-specific BMAL1 plays critical roles in metabolism, signaling, and maintenance of contractile function of the heart. *Journal of biological rhythms*. Aug.2014 29:257–276. [PubMed: 25238855]
13. Bray MS, Shaw CA, Moore MW, et al. Disruption of the circadian clock within the cardiomyocyte influences myocardial contractile function, metabolism, and gene expression. *Am J Physiol Heart Circ Physiol*. Feb.2008 294:H1036–1047. [PubMed: 18156197]
14. Zhang S. Isolation and characterization of I(Kr) in cardiac myocytes by Cs<sup>+</sup> permeation. *American journal of physiology Heart and circulatory physiology*. Mar.2006 290:H1038–1049. [PubMed: 16227343]
15. Guo J, Massaeli H, Li W, Xu J, Luo T, Shaw J, Kirshenbaum LA, Zhang S. Identification of IKr and its trafficking disruption induced by probucol in cultured neonatal rat cardiomyocytes. *The Journal of pharmacology and experimental therapeutics*. Jun.2007 321:911–920. [PubMed: 17377062]

16. Guo J, Massaeli H, Xu J, Jia Z, Wigle JT, Mesaeli N, Zhang S. Extracellular K<sup>+</sup> concentration controls cell surface density of IKr in rabbit hearts and of the HERG channel in human cell lines. *The Journal of clinical investigation*. Sep.2009 119:2745–2757. [PubMed: 19726881]
17. Zhang X, Patel SP, McCarthy JJ, Rabchevsky AG, Goldhamer DJ, Esser KA. A non-canonical E-box within the MyoD core enhancer is necessary for circadian expression in skeletal muscle. *Nucleic Acids Research*. Apr 1.2012 40:3419–3430. [PubMed: 22210883]
18. Schroder EA, Burgess DE, Manning CL, Zhao Y, Moss AJ, Patwardhan A, Elayi CS, Esser KA, Delisle BP. Light phase-restricted feeding slows basal heart rate to exaggerate the type-3 long QT syndrome phenotype in mice. 3072014
19. Mitchell GF, Jeron A, Koren G. Measurement of heart rate and Q-T interval in the conscious mouse. *Am J Physiol*. Mar.1998 274:H747–751. [PubMed: 9530184]
20. Hughes ME, Hogenesch JB, Kornacker K. JTK\_CYCLE: An Efficient Nonparametric Algorithm for Detecting Rhythmic Components in Genome-Scale Data Sets. *Journal of Biological Rhythms*. Oct 1.2010 25:372–380. [PubMed: 20876817]
21. Pizarro A, Hayer K, Lahens NF, Hogenesch JB. CircaDB: a database of mammalian circadian gene expression profiles. *Nucleic Acids Research*. Jan 1.2013 41:D1009–D1013. [PubMed: 23180795]
22. Sanguinetti MC, Jiang C, Curran ME, Keating MT. A mechanistic link between an inherited and an acquired cardiac arrhythmia: HERG encodes the IKr potassium channel. *Cell*. Apr 21.1995 81:299–307. [PubMed: 7736582]
23. Sanguinetti MC, Tristani-Firouzi M. hERG potassium channels and cardiac arrhythmia. *Nature*. 2006; 440:463–469. 03/23/print. [PubMed: 16554806]
24. Zhang EE, Kay SA. Clocks not winding down: unravelling circadian networks. *Nat Rev Mol Cell Biol*. 2010; 11:764–776. 11//print. [PubMed: 20966970]
25. Merri M, Moss AJ, Benhorin J, Locati EH, Alberti M, Badilini F. Relation between ventricular repolarization duration and cardiac cycle length during 24-hour Holter recordings. Findings in normal patients and patients with long QT syndrome. *Circulation*. May 1.1992 85:1816–1821. [PubMed: 1572038]

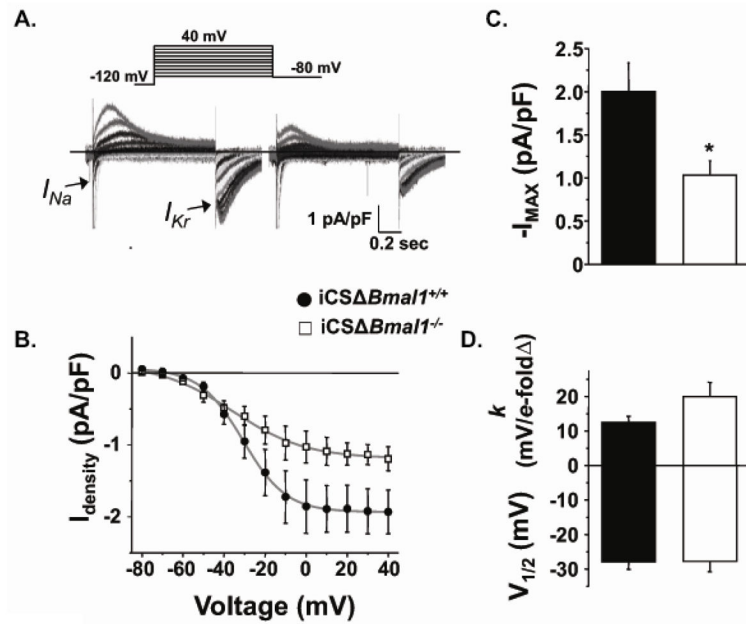
### CLINICAL PERSPECTIVES

Is there a diurnal rhythm in the QTc interval? For over 25 years clinician scientists have been working to answer this question. It is an important question because if there is a circadian rhythm in the QTc interval, then the time-of-day it is measured could influence its utility in determining of whether patients can safely tolerate drugs known to affect the QTc interval or in the diagnosis of the congenital long QT syndrome (LQTS). The general consensus is that, if there is a diurnal variation in the QTc interval, it is very small. This observation has also been confirmed in mice. However, data in our manuscript now suggests that an internal time-keeping mechanism intrinsic to cardiomyocytes directly contributes to buffering against daily changes in the QTc interval. If the intrinsic timing-keeping mechanism in the heart is disrupted, then the diurnal change in the QTc interval is amplified by as much as 10%. In humans, this would translate into about a 40 ms difference in the QTc measurement! In other words, although most patients might not normally show a large circadian flux in their QTc interval, they might if the internal clock mechanism in the heart is disrupted. Instead of simply taking single snaps shots of ventricular repolarization, taking diurnal temporal changes into consideration may better delineate at-risk patients for arrhythmias. Our findings may ultimately help physicians to increase their clinical suspicion for prolonged QT syndrome or predict the QT prolongation while initiating a QT prolonging drug.



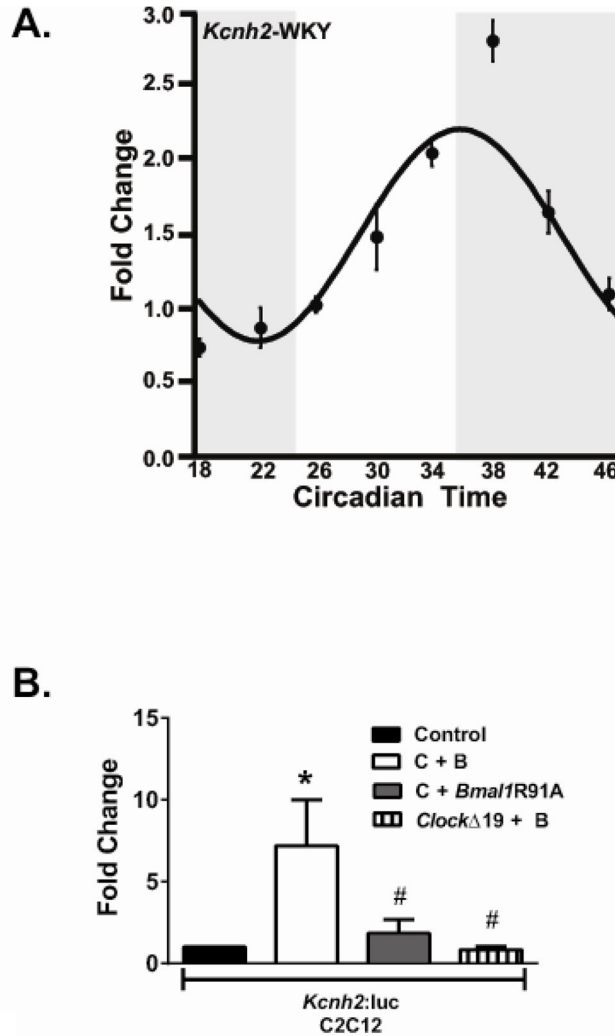
**Figure 1. The cardiomyocyte molecular clock regulates the circadian expression of *Kcnh2* transcripts as well as the expression of several  $K^+$  channel gene transcripts that are not circadian**

**A)** Shown are the JTK\_CYCLE best fit data for the mRNA expression profiles for the  $K^+$  channel genes *Kcnd2* and *Kcnh2* from iCS *Bmal1*<sup>+/+</sup> (solid circles) and iCS *Bmal1*<sup>-/-</sup> (grey squares) mouse hearts (n=3–4 animals per time point). The dark and light bars on the graph represent extrapolated subjective day and night as defined by Circadian Time (CT) according to the prior L:D cycle before release into DD. JTK\_CYCLE statistics were used to determine if the expression pattern was circadian. The JTK\_CYCLE calculated *p* value for *Kcnd2* and *Kcnh2* in the iCS *Bmal1*<sup>+/+</sup> mice is 0.003 and 7.02E-05 respectively, and the JTK\_CYCLE calculate *p* value for *Kcnd2* and *Kcnh2* in the iCS *Bmal1*<sup>-/-</sup> mice is 0.0095 and 0.060 respectively. Channels with JTK\_CYCLE *p* values less than 0.05 were considered circadian in expression. **B)** Shown is the percent change in the mRNA transcript over a 24-hour period for *Kcnd2*, *Kcnip2*, *Kcna5*, *Kcnb1*, *Kcnj2*, *Kcnq1*, and *Kcnh2* in the iCS *Bmal1*<sup>-/-</sup> mice compared to iCS *Bmal1*<sup>+/+</sup> mice.



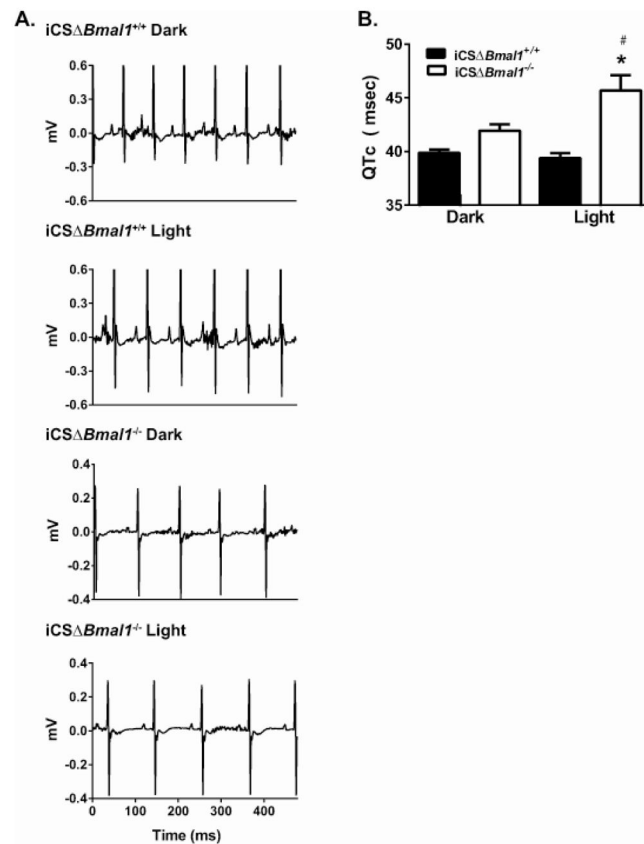
**Figure 2. Disruption of BMAL1 signaling reduces  $I_{Kr}$**

**A)** Shown are representative families of currents recorded from iCS  $Bmal1^{+/+}$  or iCS  $Bmal1^{-/-}$  ventricular cardiomyocytes (isolated from mice after 58 hours in constant darkness). The currents were recorded by pre-pulsing cells from -80 to 40 mV in 10 mV increments, followed by a test-pulse to -80 mV. The peak  $I_{Na}$  measured during the initial pre-pulse is cropped for presentation purposes. **B)** The peak inward  $I_{Kr}$  measured during the test-pulse is plotted as a function of the pre-pulse potential and the data are described using a Boltzmann equation (grey line). **C–D)** The mean Boltzmann data from the fits to the individual cells for maximally activated  $I_{Kr}$  ( $I_{MAX}$ , pA/pF), the slope factor ( $k$ , mV/ $e$ -fold change), and the midpoint potential for half-maximal activation ( $V_{1/2}$ , mV) are shown for iCS  $Bmal1^{+/+}$  (solid bars) and iCS  $Bmal1^{-/-}$  (open bars) mice ( $n=11-13$  cells,  $*P<0.05$ ).



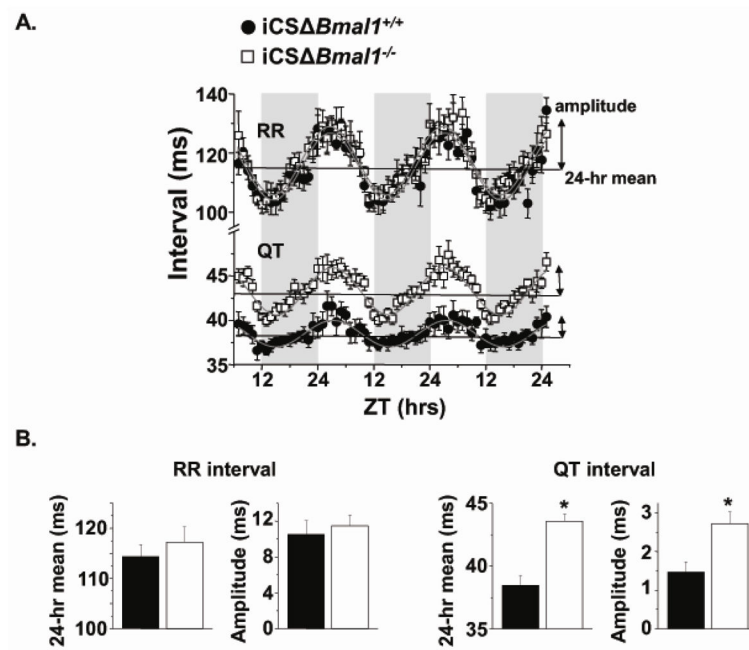
**Figure 3. *Kcnh2* transcripts follow a circadian pattern of expression in rat hearts and the human *Kcnh2* promoter is transactivated by the co-expression of BMAL1 and CLOCK**

**A)** The circadian expression profile for *Kcnh2* in the WKY rat heart is shown ( $n=4$  animals per time point). Shaded and light regions represent subjective dark and light cycle. **B)** BMAL1 and CLOCK transactivate the human *Kcnh2* promoter reporter. Luciferase assay results of transfection experiments using the h*Kcnh2* reporter gene in C2C12 myoblasts ( $n = 6-10$ /condition). CLOCK (C) and BMAL1 (B) significantly transactivate the h*Kcnh2* reporter gene (solid bars) relative to control transfections (open bars). Activation of the h*Kcnh2* reporter is significantly diminished when *Bmal1*R91A was overexpressed with CLOCK (gray bar) or *Clock* 19 was overexpressed with BMAL1 (vertical striped bar). Values are expressed as means  $\pm$  SE. \* $P < 0.05$  compared with the h*Kcnh2* reporter gene; # $P < 0.05$  compared with h*Kcnh2* reporter gene + CLOCK + BMAL.



**Figure 4. iCS  $Bmal1^{-/-}$  mice have a prolonged QTc intervals during the light-phase**  
**A)** Shown are representative ECG traces measured during the dark and light from iCS  $Bmal1^{+/+}$  or iCS  $Bmal1^{-/-}$  mice. **B)** QTc intervals measured at peak and trough RR interval hours during the light- and dark-phases from iCS  $Bmal1^{-/-}$  (open bars) or iCS  $Bmal1^{+/+}$  mice (solid bars) (n=6–7 animals, \*P<0.05 compared to dark values; #<0.05 compared to iCS  $Bmal1^{+/+}$ ).





**Figure 5.  $iCS\ Bmal1^{-/-}$  mice have a larger circadian rhythm mean and amplitude in their uncorrected QT intervals**

**A)** ECGs were continuously measured using telemetry for ~3 days and the hourly RR or uncorrected QT intervals were plotted as a function of ZT for  $iCS\ Bmal1^{+/+}$  (solid circles) and  $iCS\ Bmal1^{-/-}$  (open squares) mice ( $n=6-7$ ). The shaded and light regions represent the dark and light, respectively. The grey line is a non-linear sinusoidal fit to the data. **B)** Shown are the average circadian rhythm mean and the amplitude for the RR interval and QT interval calculated from the sinusoidal fits to individual  $iCS\ Bmal1^{+/+}$  (solid bars) or  $iCS\ Bmal1^{-/-}$  (open bars) mouse data ( $n=6-7$  each,  $*P<0.05$ )

**Table 1**  
**Disruption of the cardiomyocyte molecular clock selectively increases the mean and amplitude of the circadian rhythm in the QT interval**

The table shows the circadian RR or QT interval parameters from iCS *Bmal1*<sup>+/+</sup> and iCS *Bmal1*<sup>-/-</sup> mice before (Pre-) and after (Post-) vehicle or tamoxifen treatment.

RR or QT interval	Pre-iCS <i>Bmal1</i> <sup>+/+</sup> vehicle	Post-iCS <i>Bmal1</i> <sup>+/+</sup> vehicle	Pre-iCS <i>Bmal1</i> <sup>-/-</sup> tamoxifen	Post-iCS <i>Bmal1</i> <sup>-/-</sup> tamoxifen
RR midline (ms)	111.7 ± 1.8	114.3 ± 2.4	108.3 ± 3.1	117.3 ± 3.1
RR amplitude (ms)	25.5 ± 0.9	10.5 ± 1.5	8.4 ± 1.2	11.5 ± 1.2
RR phase (hrs)	2.3 ± 0.7	2.6 ± 0.5	3.0 ± 0.6	2.4 ± 0.3
RR period (hrs)	25.5 ± 0.9	24.9 ± 0.6	24.3 ± 0.4	24.2 ± 0.1
QT midline (ms)	38.8 ± 0.9	38.5 ± 0.7	38.8 ± 1.1	43.6 ± 0.6*
QT amplitude (ms)	1.2 ± 0.1	1.4 ± 0.3	1.6 ± 0.4	2.7 ± 0.3*
QT phase (hrs)	3.9 ± 0.9	2.9 ± 0.9	4.9 ± 0.4	3.2 ± 0.5
QT period (hrs)	23.9 ± 0.3	24.4 ± 0.3	23.7 ± 0.2	24.0 ± 0.1
N	7	7	6	6

\* p<0.05 compared to pre-iCS *Bmal1*<sup>+/+</sup>, post-iCS *Bmal1*<sup>+/+</sup> and pre-iCS *Bmal1*<sup>-/-</sup>