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## Disruption of the circadian clock within the cardiomyocyte influences myocardial contractile function, metabolism, and gene expression

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**Bray MS, Shaw CA, Moore MW, Garcia RA, Zanquetta MM, Durgan DJ, Jeong WJ, Tsai JY, Bugger H, Zhang D, Rohrwasser A, Rennison JH, Dyck JR, Litwin SE, Hardin PE, Chow CW, Chandler MP, Abel ED, Young ME.** Disruption of the circadian clock within the cardiomyocyte influences myocardial contractile function, metabolism, and gene expression. *Am J Physiol Heart Circ Physiol* 294: H1036–H1047, 2008. First published December 21, 2007; doi:10.1152/ajpheart.01291.2007.— Virtually every mammalian cell, including cardiomyocytes, possesses an intrinsic circadian clock. The role of this transcriptionally based molecular mechanism in cardiovascular biology is poorly understood. We hypothesized that the circadian clock within the cardiomyocyte influences diurnal variations in myocardial biology. We, therefore, generated a cardiomyocyte-specific circadian clock mutant (CCM) mouse to test this hypothesis. At 12 wk of age, CCM mice exhibit normal myocardial contractile function *in vivo*, as assessed by echocardiography. Radiotelemetry studies reveal attenuation of heart rate diurnal variations and bradycardia in CCM mice (in the absence of conduction system abnormalities). Reduced heart rate persisted in CCM hearts perfused *ex vivo* in the working mode, highlighting the intrinsic nature of this phenotype. Wild-type, but not CCM, hearts exhibited a marked diurnal variation in responsiveness to an elevation in workload (80 mmHg plus 1  $\mu$ M epinephrine) *ex vivo*, with a greater increase in cardiac power and efficiency during the dark (active) phase vs. the light (inactive) phase. Moreover, myocardial oxygen consumption and fatty acid oxidation rates were increased, whereas cardiac efficiency was decreased, in CCM hearts. These observations were associated with no alterations in mitochondrial content or structure and modest mitochondrial dysfunction in CCM hearts. Gene expression microarray analysis identified 548 and 176 genes in atria and ventricles, respectively, whose normal diurnal expression patterns were altered in CCM mice. These studies suggest that the cardiomyocyte circadian clock influences myocardial contractile function, metabolism, and gene expression.

bradycardia; carbohydrate; chronobiology; epinephrine; fatty acids

OVER THE COURSE OF A NORMAL 24-h day, the environment of, and the demands placed on, cells, organs, and organisms

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fluctuate dramatically. Many organisms have, therefore, evolved circadian clocks, which are intracellular molecular mechanisms that allow individual cells to perceive the time of day (11). In doing so, circadian clocks confer the selective advantage of anticipation, enabling both rapid and appropriate responses to environmental stimuli upon their onset. Almost every mammalian cell possesses an intrinsic circadian clock, a transcriptionally based molecular mechanism capable of regulating multiple cellular functions. In terms of the cardiovascular system, circadian clocks have been characterized within multiple cell types, including cardiomyocytes (7, 42); however, little is known regarding the influence of intramyocellular circadian clocks on cardiovascular physiology and pathophysiology. This is particularly relevant, given the possibility that impairment of the circadian clock within the cardiomyocyte may significantly alter cardiac function, pathogenesis of cardiovascular disease (CVD), and/or treatment strategies during CVD states (e.g., chronopharmacology) (42).

To date, experiments investigating the influence of altered circadian clock function *in vivo* have primarily involved manipulation of the light-dark cycle and/or genetic ablation of circadian clocks in a ubiquitous manner (24, 36). Experimental strategies using such global manipulations influence both central and peripheral circadian clocks within the organism, as well as virtually every neurohumoral factor investigated to date (24, 36, 40). The most extensively characterized of these models is the *Clock* mutant mouse, in which chemical mutagenesis-induced truncation of the native *Clock* gene resulted in a dominant-negative CLOCK (dnCLOCK) mutant protein lacking the transactivation domain of this transcription factor (termed CLOCK <sup>$\Delta$ 19</sup>, due to loss of *exon 19*). We recently generated a cardiomyocyte-specific circadian clock mutant (CCM) mouse, through MHC- $\alpha$  promoter-driven expression of the CLOCK <sup>$\Delta$ 19</sup> protein within cardiomyocytes (9). We employed this dominant-negative approach, in contrast to a tissue-

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specific CLOCK knockout approach, due to the marked redundancy within the mammalian circadian clock mechanism. Ubiquitous CLOCK null mice exhibit normal circadian rhythms at multiple biological levels, due to compensation by the CLOCK homolog neuronal Per-Arnt-Sim domain protein 2 (6). In our CCM mice, the circadian clock within the heart is severely attenuated in a sex-independent manner, as demonstrated by attenuation of clock component and output gene circadian oscillations, without affecting circadian clocks within extracardiac tissues (9). Using this model, our laboratory has recently shown that the circadian clock within the cardiomyocyte is essential for responsiveness of the heart to fatty acids (9).

A global understanding of the role of the circadian clock within the cardiomyocyte is currently lacking. As such, the purpose of the present study was to characterize cardiac function, metabolism, and gene expression in CCM mice, thereby identifying novel potential roles for the circadian clock within the cardiomyocyte. We find that disruption of this molecular mechanism within the cardiomyocyte influences diurnal variations in heart rate, responsiveness of the heart to changes in workload, myocardial metabolism, as well as a plethora of myocardial genes known to influence transport, transcription, signal transduction, protein turnover, and metabolism.

## MATERIALS AND METHODS

**Animals.** Male CCM (on the FVB/N background) and littermate controls were housed at the Centers for Comparative Medicine at Baylor College of Medicine (ex vivo heart perfusion, mitochondrial DNA content, and gene expression studies), at the University of Utah (echocardiographic, radiotelemetric, and electron microscopy studies), or at Case Western Reserve University (isolated mitochondrial and enzymatic studies). All studies were approved by the respective local Institutional Animal Care and Use Committees. All mice were housed under controlled conditions ( $23 \pm 1^\circ\text{C}$ ; 12:12-h light-dark cycle), and received standard laboratory chow and water ad libitum. At least 21 days before they were killed, mice were housed in pairs in a separate environment-controlled room, within which a strict 12:12-h light-dark cycle regime was enforced [lights on at 7 AM; zeitgeber time (ZT) 0]. The light-dark cycle was maintained throughout these studies, facilitating elucidation of the potential roles for the cardiomyocyte circadian clock under physiological conditions. As such, diurnal variations were investigated.

**Echocardiographic and radiotelemetric studies.** In vivo myocardial contractile function was assessed in anesthetized mice (isoflurane) using echocardiography, as described previously (21). Ambulatory heart rate and blood pressure, as well as activity, were continuously assessed by radiotelemetry, as described previously (3). Radiotelemetric devices were surgically implanted in 11-wk-old wild-type and CCM mice, which were allowed to recover from this intervention for 1 wk before a 2-wk data collection period (i.e., 12–14 wk of age). ECG telemetry was performed in a similar manner, in which radiotelemetric devices were implanted in 11-wk-old mice 1 wk before initiation of data collection.

**Isolated working mouse heart perfusions.** Isolated working mouse heart perfusions were utilized to investigate diurnal variations in myocardial metabolism and contractile function ex vivo, as described previously (10). Hearts were perfused in the working mode in a non-recirculating manner with Krebs-Henseleit bicarbonate buffer containing 5 mM glucose, 0.4 mM oleate conjugated to 3% BSA (fraction V, fatty acid free; dialyzed), 10  $\mu\text{U/ml}$  insulin, and tracer amounts of [ $^3\text{H}$ ]glucose (40  $\mu\text{Ci/l}$ ) and [ $^3\text{H}$ ]oleate (60  $\mu\text{Ci/l}$ ). After 30 min of normal workload perfusion conditions (11.5 mmHg preload, 50 mmHg afterload), hearts were perfused for an additional 30 min under high workload conditions (11.5 mmHg H<sub>2</sub>O preload, 80

mmHg afterload plus 1  $\mu\text{M}$  epinephrine). Cardiac power, efficiency, heart rate, developed pressure, and rate pressure product, as well as rates of oxygen consumption [myocardial oxygen consumption ( $\text{M}\dot{\text{V}}\text{O}_2$ )], exogenous oleate oxidation, exogenous glucose oxidation, net lactate release (i.e., lactate derived from both extracellular glucose, as well as intracellular glycogen),  $^{14}\text{C}$ -labeled lactate release, and endogenously derived lactate release were determined as described previously (1, 8, 10). Data presented for this study are those obtained during the last 10 min of both the low and high workload conditions. These steady-state rates expose the intrinsic properties of the myocardium in the absence of acute influences by extrinsic (i.e., neurohumoral) factors (44).

**Mitochondrial studies.** Mitochondrial DNA content was measured through PCR of mitochondrial (cytochrome *b*) and nuclear ( $\beta$ -actin) specific genes, as described previously (2). Mitochondrial number and volume density were determined from electron micrographs, as described previously (2). Citrate synthase and medium chain acyl-CoA dehydrogenase (MCAD) activities were measured spectrophotometrically, as described previously (27). For determination of mitochondrial function, cardiac subsarcolemmal (SSM) and intramyofibrillar (IFM) mitochondria were isolated from wild-type and CCM hearts using the procedure of Palmer et al. (26), except that a modified Chappell-Perry buffer (containing 100 mM KCl, 50 mM MOPS, 1 mM EGTA, 5 mM  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ , and 1 mM ATP, pH 7.4 at  $4^\circ\text{C}$ ) was used for isolation of both mitochondrial populations. Mice were anesthetized (isoflurane), and the left ventricle was excised and subsequently placed in KME (100 mM KCl, 50 mM MOPS, internal salt, and 0.5 mM EGTA). The IFM were harvested following treatment of skinned fibers with 5 mg/g wet wt trypsin for 10 min at  $4^\circ\text{C}$  (22). Mitochondrial protein concentration was determined by the Lowry method using bovine serum albumin as a standard.

Oxygen consumption in SSM and IFM was measured using a Clark-type oxygen electrode (Strathkelvin) at  $30^\circ\text{C}$  (35). Mitochondria were incubated in a solution consisting of 80 mM KCl, 50 mM MOPS, 1 mM EGTA, 5 mM  $\text{KH}_2\text{PO}_4$ , and 1 mg/ml bovine serum albumin at pH 7.4. The rate of oxidative phosphorylation was measured using the complex-specific substrates glutamate (complexes I, III, and IV), succinate (complexes II, III, and IV), durohydroquinone (complexes III and IV), and *N,N,N',N'*-tetramethyl-*p*-phenylenediamine-ascorbate (complex IV) (29, 30); durohydroquinone was a kind gift from Dr. Charles Hoppel (Case Western Reserve University). Mitochondrial respiration was also measured using pyruvate plus malate and acetylcarnitine plus malate, which assess pyruvate dehydrogenase and carnitine-acetyltransferase activities, respectively (29). Lipid substrates carnitine-dependent octanoyl-CoA plus malate, carnitine-dependent palmitoyl-CoA plus malate, and palmitoylcarnitine plus malate were used to assess carnitine palmitoyltransferase I, palmitoyltransferase II, and carnitine-acylcarnitine translocase activities (17). State 3 (ADP stimulated) respiration, state 4 (ADP limited) respiration, respiratory control ratio (RCR) (state 3/state 4, an indicator of mitochondrial coupling), and ADP/O ratio (ratio of oxygen consumed following addition of known amount of ADP) were determined, as described previously (4, 12). Uncoupled respiration, initiated by addition of the uncoupler dinitrophenol, was used to assess the phosphorylation apparatus, which is composed of adenine nucleotide translocase,  $\text{F}_1\text{F}_0$  ATPase, and the pyruvate transporter (29).

**Microarray analysis.** Microarray gene expression studies were performed using mouse Ref-6 BeadChips and the BeadStation system from Illumina (San Diego, CA). Atrial and ventricular tissue samples were collected from wild-type and CCM animals every 3 h for a period of 24 h ( $n = 4$  samples per time point; 128 samples total), and RNA was subsequently extracted from each sample using standard procedures (5). Microarrays were performed according to the manufacturer's guidelines. Total RNA was converted to cDNA by reverse transcription using ArrayScript reverse transcriptase and T7-(dT)<sub>24</sub> primers, followed by second-strand synthesis to generate double-stranded cDNA (Ambion). After purification, the cDNA was con-

verted to biotin-labeled cRNA, hybridized to the BeadChip (Illumina), and stained with streptavidin-Cy3 for visualization. The mouse Ref-6 BeadChips contain sequences representing ~46,000 curated genes and putative expressed sequence tags, with an average of 30 replicates for each gene/transcript on each BeadChip. Quality standards for hybridization, labeling, staining, background signal, and basal level of housekeeping gene expression were verified for all BeadChips.

After the probe arrays were scanned, the resulting images were first analyzed using the BeadStudio software (Illumina, San Diego, CA), which calculates the mean fluorescence signal across all replicates of each gene/transcript, along with a probability that the mean signal for each gene/transcript on the chip is greater than background (i.e., detection score). Genes/transcripts were defined as being significantly expressed above background when the 75th percentile of each gene's detection score met or exceeded 0.9. The expression data were then normalized within centiles of the distribution of gene expression values. Identification of genes exhibiting significant oscillations in expression was next performed using the method described by Straume (33). Separate analyses were performed for the atrium and ventricle tissues. Rhythmicity of gene expression for each Illumina probe set was evaluated by fitting the nonlinear cosine function,  $A * \cos[(2\pi t/T) + \Phi] + C$  (where  $C$  is the mean expression of the genes across all time,  $A$  is the amplitude of oscillation,  $\Phi$  is a phase shift,  $t$  is time and  $T$  is the period of oscillation). In addition, Fisher's G statistic for spectral power density was calculated for each gene using the time point means to make the calculation. Fits were performed in the R statistical programming environment using the nonlinear least squares (NLS) method to identify the best amplitude, period, and phase shift parameters and to minimize the sum of squared deviations about the fitted curve for each probe set. Before NLS estimation of the periodic curve, the gene mean  $C$  was calculated as the mean of the time point means, and this value was subtracted from the data. The centered data were then subject to analysis using the R implementation of the NLS fitting procedure; this procedure searches for the global optimum of the least squares criterion. The search is accomplished by Newton-Raphson gradient-based method, which is computationally efficient compared with a more grid-based approach. To consider the significance of the oscillatory fits, we implemented a simulation procedure to parallel that which is described in the well-accepted COSOPT procedure. For each Illumina probe set, we determined the standard error of the mean at each time point. We then performed 1,000 random permutations of the time point means, on which we superimposed independent Gaussian noise with mean 0 and with variance determined by the time point standard error. The NLS best fit for each permuted data set was then calculated. The ensemble of 1,000 parameter fits for the permuted data were then compared with the true data fits to determine which genes showed significant patterns of oscillation. A gene (probe set) was considered to have a significant circadian oscillation if it fit a cosine wave function for  $p$  multiple measures corrected  $\beta$  ( $MMC\beta$ )  $< 0.05$  and Fisher  $G < 0.1$ , with a periodicity of between 20 and 28 h. The  $P$  values for both the  $MMC\beta$  parameter and Fisher  $G$  were determined using the simulation-based procedure. This criterion was evaluated through the use of 10 known circadian clock guide genes, all of which oscillated significantly in wild-type atria and ventricles, as assessed by this analytic strategy. Subsequently, a two-way ANOVA was performed to identify those oscillating genes identified as differentially expressed in CCM atria and ventricles. The two-way ANOVA was used to identify genes with shifts in mean expression (interpreted as a main effect of genotype) and with genotype-time interaction (interpreted as a phase shift in expression associated with genotype). Ontology and pathway analyses were performed to group genes from the final lists using the Onto-Tools package of ontology and pathway analysis from Wayne State University (<http://vortex.cs.wayne.edu/projects.htm>). Normalized data have been submitted to GEO archive and are available at <http://www.ncbi.nlm.nih.gov/geo/>.

**Quantitative RT-PCR.** Quantitative RT-PCR was performed using previously described methods (14, 16). Specific quantitative assays were designed from mouse sequences available in GenBank. Primer and probe sequences for *bmal1*, *dbp*, *per3*, *dgat2*, and *adpn* Taqman assays have been published previously (9, 45), whereas those for *dec1*, *e4bp4*, *mcip1*, *pbef1*, *pik3r1*, and *prkar1a* are presented in Supplemental Table 1. (The online version of this article contains supplemental data.) Standard RNA was made for all assays by the T7 polymerase method (Ambion, Austin, TX), using total RNA isolated from mouse hearts; the use of standard RNA allows absolute quantification of gene expression. The correlation between the threshold cycle (the number of PCR cycles required for the fluorescent signal to reach a detection threshold) and the amount of standard was linear over at least a five-log range of RNA for all assays (data not shown). Quantitative RT-PCR data are represented as mRNA molecules per nanogram total RNA.

**Statistical analysis of nonmicroarray data.** Telemetry data were analyzed using a one-way ANOVA with repeated measures. One-way ANOVA was used to compare peak and trough values by genotype. For analyses of main effects of group (e.g., genotype, workload) and time, two-way ANOVA was performed, followed by post hoc pairwise comparisons of time points by genotype. Stata version 8.0 (Stata, San Antonio, TX) was used to perform two-way ANOVA to investigate the main effects of intervention and time. A full model, including second-order interactions, was conducted for each experiment. Significant differences were determined using Type III sums of squares. The null hypothesis of no model effects was rejected at  $P < 0.05$ .

## RESULTS

**In vivo assessment of cardiovascular function.** In vivo cardiac function was evaluated at ZT6 in 12-wk-old anaesthetized wild-type and CCM mice by echocardiography (Table 1). Of the parameters measured, fractional shortening, left ventricular ejection fraction, and biventricular mass were significantly altered (all elevated) in CCM mice. Next, radiotelemetric studies were performed, for continuous measurement of physical activity, systolic blood pressure (SBP), diastolic blood pressure (DBP), mean arterial pressure (MAP), and heart rate in conscious mice. Relative to wild-type mice, CCM mice exhibited a lower heart rate during both the light and dark

Table 1. Echocardiographic analysis of anesthetized wild-type and CCM (12 wk of age) mice at zeitgeber time 6 (i.e., middle of the light phase)

|               | Wild Type   | CCM          |
|---------------|-------------|--------------|
| LVDD, cm      | 0.400±0.014 | 0.395±0.011  |
| LVDs, cm      | 0.235±0.011 | 0.208±0.009  |
| IVDs, cm      | 0.082±0.004 | 0.097±0.005  |
| LVPWd, cm     | 0.085±0.002 | 0.100±0.007  |
| FS, %         | 42.34±1.75  | 47.32±1.48*  |
| LVEF          | 0.797±0.015 | 0.853±0.013* |
| LVOTvti, cm   | 3.67±0.18   | 4.43±0.18    |
| HR, beats/min | 462±32      | 399±16       |
| CO, ml/min    | 13.22±0.89  | 13.85±0.78   |
| Mass, g       | 0.127±0.006 | 0.153±0.009* |

Data are shown as means  $\pm$  SE for 6 separate mice per group. CCM, cardiomyocyte-specific circadian clock mutant; LVDD, left ventricular diameter in diastole; LVDs, left ventricular diameter in systole; IVDs, interventricular septum thickness in systole; LVPWd, left ventricular posterior wall thickness in diastole; FS, fractional shortening; LVEF, left ventricular ejection fraction; LVOTvti, left ventricular outflow tract velocity time integral (stroke volume index); HR, heart rate; CO, cardiac output. \* $P < 0.05$ , wild-type vs. CCM.

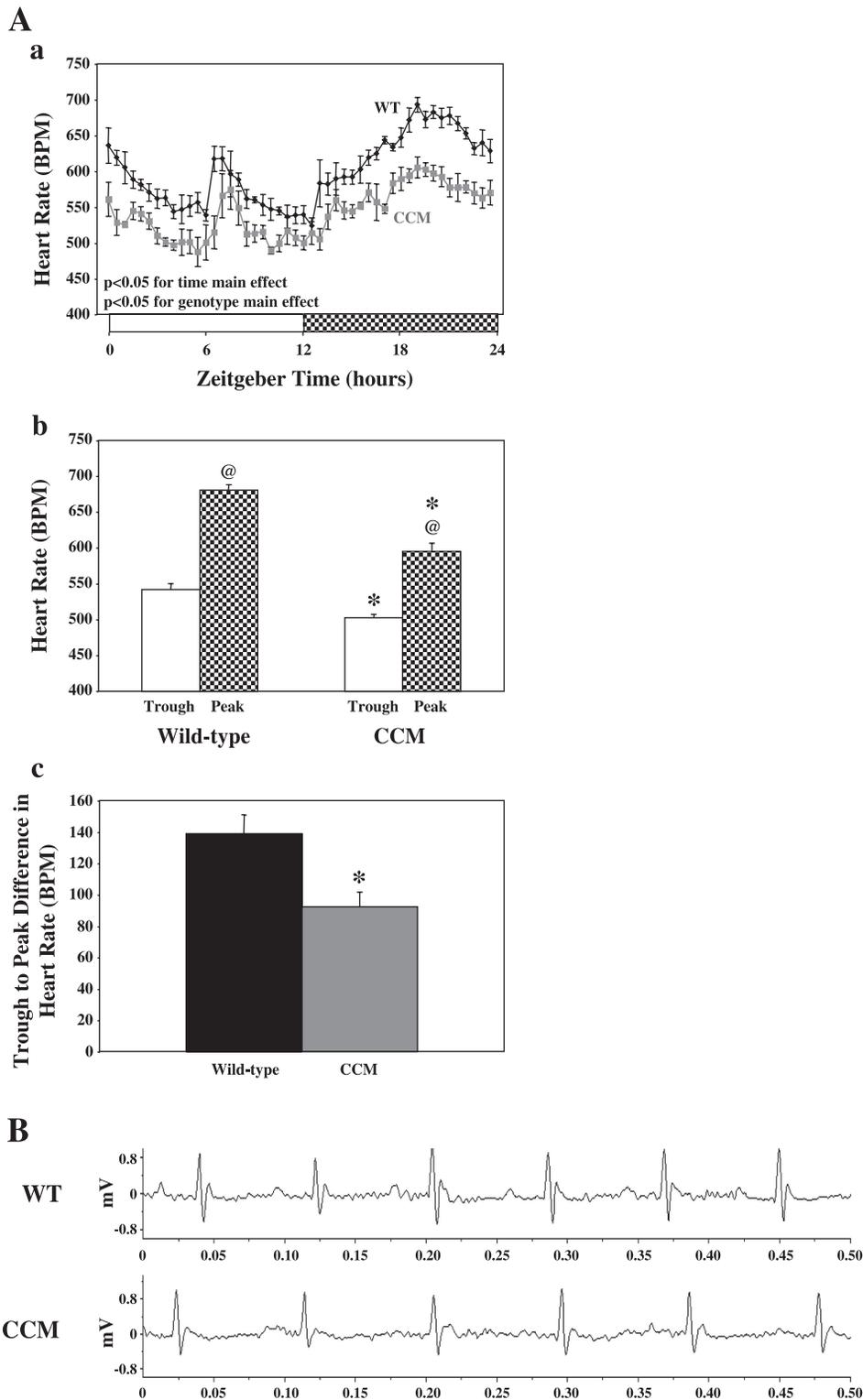


Fig. 1. Heart rate (A) and ECG (B) radiotelemetric analysis of wild-type (WT) vs. cardiomyocyte-specific circadian clock mutant (CCM) mice. Radiotelemetric devices were implanted in mice at 11 wk of age, and data collection was initiated at 12 wk of age and continued continuously for a 2-wk period. *Aa*: data across a 24-h period, at 0.5-h intervals. As indicated, significant main effects of genotype and time were observed ( $P < 0.05$ ). *Ab*: trough [average of zeitgeber time (ZT) 10 through ZT12] and peak (average of ZT18 through ZT20) values. *Ac*: trough-to-peak differences (i.e., amplitude of oscillation). *B*: representative ECG tracings for WT and CCM mice. Data are shown as means  $\pm$  SE for 4 mice per group. BPM, beats/min. For comparisons of peak/trough values: \* $P < 0.05$ , WT vs. CCM; @ $P < 0.05$ , trough vs. peak.

phases (Fig. 1*Aa*). The decrease in heart rate was greater during the dark phase (Fig. 1*Ab*), such that CCM mice exhibit attenuated diurnal variations in this parameter (Fig. 1*Ac*). Despite the lower heart rate observed in CCM mice, comparable diurnal variations in physical activity between wild-type and CCM mice were observed; both groups exhibit increased activity during the dark phase relative to the light phase

(Supplemental Fig. 1A). Increased physical activity during the dark phase was associated with increased SBP, DBP, MAP (Supplemental Fig. 1, *B*, *C*, and *D*, respectively), and heart rate (Fig. 1A) in both wild-type and CCM mice. Similar to physical activity, no significant differences were observed for trough and peak values of SBP, DBP, or MAP between wild-type and CCM mice.

Table 2. *Electrocardiographic analysis of wild-type and CCM (12 wk of age) at zeitgeber time 6 (i.e., middle of the light phase) through radiotelemetry*

|                       | Wild Type   | CCM          |
|-----------------------|-------------|--------------|
| PQ, s                 | 0.019±0.001 | 0.020±0.001  |
| PR, s                 | 0.034±0.001 | 0.034±0.000  |
| QRS, s                | 0.018±0.001 | 0.019±0.000  |
| QT, s                 | 0.029±0.000 | 0.031±0.000  |
| QTcB, s               | 0.099±0.001 | 0.103±0.001  |
| R-R, s                | 0.083±0.001 | 0.089±0.001* |
| Heart rate, beats/min | 725±12      | 672±8*       |

Data are means ± SE for 4 separate mice per group. QTcB, QT interval corrected using Bazett's formula. \* $P < 0.05$ , wild-type vs. CCM.

Conscious ECG telemetry was performed to determine the basis for the bradycardia that develops in CCM mice. Table 2 and Fig. 1B show that the only abnormality observed was an increase in the R-R interval, which is consistent with sinus bradycardia. All other intervals were normal, effectively ruling out significant conduction system abnormalities.

*Ex vivo assessment of myocardial contractile function at normal and high workloads.* We utilized the isolated working mouse heart preparation to investigate diurnal variations in heart rate for wild-type vs. CCM littermates (11 wk of age), thereby eliminating the acute influence of neurohumoral factors. Figure 2Aa shows that CCM hearts exhibit decreased heart rate, relative to wild-type hearts, when perfused *ex vivo* under normal workload conditions, independent of the time of day. These experiments suggest that the circadian clock within the cardiomyocytes of the sinoatrial node may influence pacemaker function.

The isolated working mouse heart preparation was utilized further to reveal differences in diurnal contractile function between wild-type and CCM hearts, in the absence of acute neurohumoral influences. Developed pressure and rate pressure product do not exhibit a significant time of day dependence in wild-type or CCM mouse hearts perfused under normal workload conditions (Fig. 2, Ba and Ca); however, rate pressure product is significantly reduced in CCM hearts (compared with wild-type hearts), independent of the time of day (Fig. 2Ca), consistent with the observed bradycardia (Fig. 2Aa). Similar to previously published *in vivo* studies (25, 39), Fig. 2Da shows that wild-type hearts perfused under normal workload conditions exhibit a significant diurnal variation in cardiac power (a derivative of cardiac output), peaking during the dark (active) phase (i.e., 1.3-fold higher at ZT18 vs. ZT0). In contrast, CCM hearts do not exhibit a diurnal variation in cardiac power (Fig. 2Da).  $\dot{M}\dot{V}O_2$  did not exhibit a significant diurnal variation in either wild-type or CCM hearts during normal workload conditions (Fig. 2Ea). However, independent of the time of day, CCM hearts display higher rates of oxygen consumption compared with wild-type hearts (Fig. 2Ea). Wild-type, but not CCM hearts, exhibit increased cardiac efficiency during the dark (active) phase (i.e., 1.3-fold higher at ZT18 vs. ZT6; Fig. 2Fa). CCM hearts have lower cardiac efficiency relative to wild-type littermate hearts (independent of the time of day; Fig. 2Fa).

We next tested the hypothesis that the circadian clock within the cardiomyocyte allows the heart to anticipate circadian rhythms in workload (which is usually increased during the

dark phase for the rodent). To model increased physical activity, we simultaneously stimulated the heart with epinephrine and increased the afterload *ex vivo*, as described previously (15) (note that *ex vivo* perfused hearts are in permanent parasympathetic withdrawal). Figure 2Ab shows a persistence of bradycardia in CCM mice at this high workload. Developed pressure and rate pressure product did not exhibit a significant diurnal variation in wild-type (or CCM) mouse hearts during high workload conditions (Fig. 2, Bb and Cb). Figure 2Db shows a marked diurnal variation in the responsiveness of wild-type hearts to an increased workload, in terms of cardiac power, with greatest responsiveness observed during the dark (active) phase (e.g., 1.7-fold higher at ZT18 vs. ZT12). This rhythm in responsiveness to increased workload was absent for CCM hearts. Neither wild-type nor CCM hearts exhibited a significant diurnal variation in  $\dot{M}\dot{V}O_2$  during high workload conditions (Fig. 2Eb). However, cardiac efficiency exhibited a diurnal variation in wild-type, but not CCM, mouse hearts, peaking during the dark (active) phase (i.e., ZT18; Fig. 2Fb).

*Ex vivo assessment of myocardial metabolism at normal and high workloads.* Due to known close relationships between myocardial contractile function and metabolism (34), as well as the suggestion that peripheral circadian clocks likely influence cellular metabolism (41, 42), myocardial metabolic fluxes were measured in wild-type and CCM hearts *ex vivo*, at both normal and high workloads. When perfused at a normal workload, neither rates of exogenous oleate oxidation, exogenous glucose oxidation, nor lactate release rates (net,  $^{14}C$ -labeled, endogenously derived) exhibited a time-of-day dependence in wild-type hearts under normal workload conditions (Fig. 3, Aa and Ba, and Supplemental Fig. 2, Ai, Bi, and Ci). However, consistent with increased  $\dot{M}\dot{V}O_2$  (Fig. 2Ea), exogenous oleate oxidation rates are constitutively elevated in CCM vs. wild-type hearts (Fig. 3Aa), independent of the time of day.

Neither exogenous oleate nor glucose oxidation rates exhibited significant differences between wild-type and CCM hearts under high workload conditions (Fig. 3Ab and Supplemental Fig. 2Aii). In contrast, lactate release rates (net,  $^{14}C$ -labeled, and endogenously derived) exhibited biphasic oscillations in wild-type hearts, which were absent in CCM hearts (Fig. 3Bb and Supplemental Fig. 2, Bii and Cii). During high workload conditions, CCM hearts had time-of-day independent rates of lactate release that were comparable to the lowest rates (i.e., at ZT12) observed in wild-type hearts (Fig. 3Bb and Supplemental Fig. 2, Bii and Cii), consistent with decreased responsiveness of CCM hearts to the workload challenge at the level of contractile function (Fig. 2).

*Mitochondrial number, structure, and function in CCM vs. wild-type hearts.* Given the marked differences in  $\dot{M}\dot{V}O_2$ , fatty acid oxidation, lactate release, and efficiency between wild-type and CCM hearts, as well as responsiveness to increased workload, we investigated whether alterations in mitochondria potentially contribute to the phenotypes observed in CCM hearts. Mitochondrial DNA content, number, and volume density did not differ between wild-type and CCM hearts, nor did total citrate synthase activity (Supplemental Table 2). In addition, gross mitochondrial morphology was not altered in CCM (vs. wild-type) hearts (as qualitatively assessed by electron microscopy; Supplemental Fig. 3). Because SSM and IFM have been shown to respond differentially to pathophysiological conditions, we isolated both mitochondrial populations

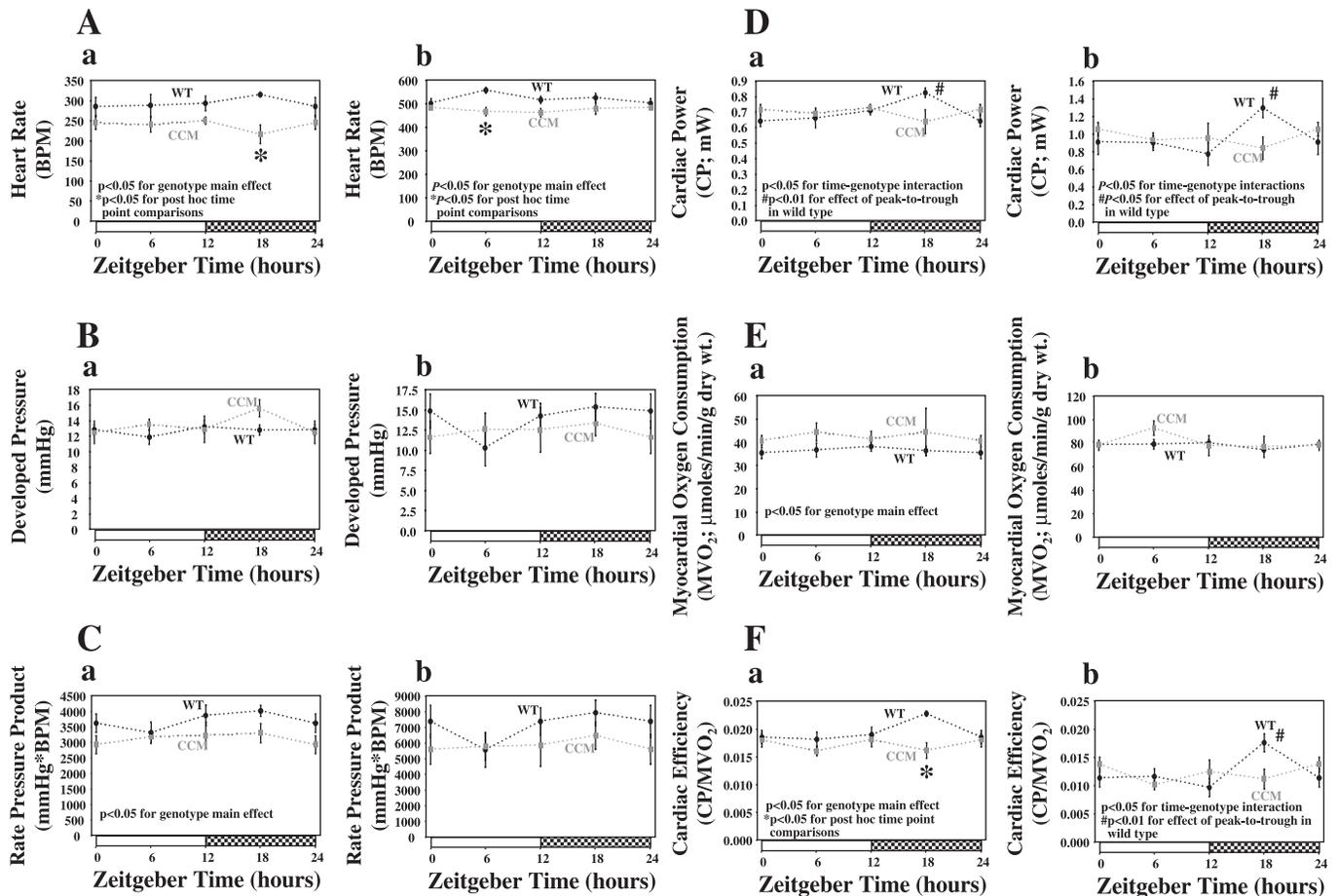


Fig. 2. Diurnal variations in contractile function of WT and CCM hearts perfused ex vivo at normal (a) and high (b) workloads. Heart rate (A), developed pressure (B), rate pressure product (C), cardiac power (CP; D), myocardial oxygen consumption ( $MVO_2$ ; E), and efficiency (F) were measured in WT and CCM hearts perfused ex vivo at ZT0, ZT6, ZT12, and ZT18, under normal (50-mmHg afterload; a) and high (80-mmHg afterload plus 1  $\mu$ M epinephrine; b) workload conditions. Mice were 11 wk of age. Data are shown as means  $\pm$  SE between 7 and 9 hearts per group. \* $P < 0.05$  for post hoc time point comparisons; # $P < 0.05$ , peak vs. trough post hoc comparison for WT hearts.

from nonperfused wild-type and CCM hearts (11 wk old). Neither total mitochondrial (i.e., SSM plus IFM) nor IFM protein yields significantly differed between CCM and wild-type hearts (Supplemental Table 2). However, SSM protein yields were significantly decreased for CCM hearts compared with wild-type hearts (Supplemental Table 2), whereas IFM protein yields tended to be increased.

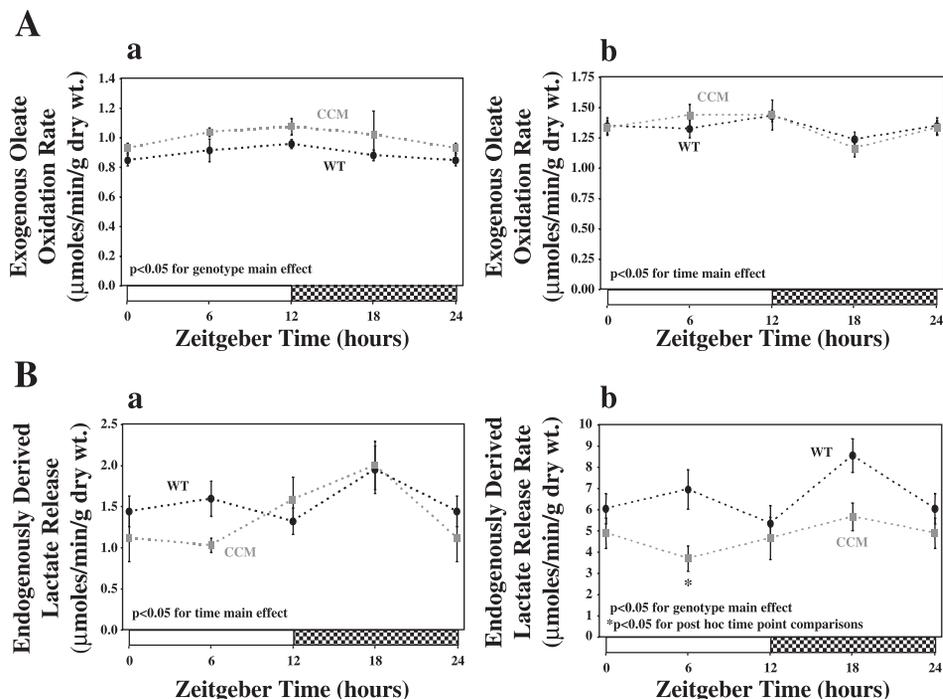
Respiratory function was investigated for SSM and IFM isolated from wild-type and CCM mouse hearts. State 3 respiration, state 4 respiration, RCR, and ADP/O were not altered in the IFM population of CCM hearts compared with wild-type hearts, using any of the substrates investigated (see Supplemental Fig. 4). In the SSM of CCM hearts, state 3 respiration was decreased using glutamate, pyruvate, palmitoylcarnitine, palmitoyl-CoA, and acetyl carnitine (Fig. 4A), as well as  $N,N,N',N'$ -tetramethyl-*p*-phenylenediamine-ascorbate ( $872 \pm 31$  vs.  $627 \pm 67$  nano atoms of oxygen (nAO)  $\cdot$  min $^{-1}$   $\cdot$  mg mitochondrial protein $^{-1}$  for wild-type vs. CCM SSM;  $P < 0.05$ ). Depressed state 3 respiration rates in the SSM population of CCM hearts were not relieved by the addition of the uncoupler dinitrophenol ( $191 \pm 33$  vs.  $115 \pm 17$  nAO  $\cdot$  min $^{-1}$   $\cdot$  mg mitochondrial protein $^{-1}$  for wild-type vs. CCM SSM, with glutamate as the sole substrate;  $P < 0.05$ ). State 4 respiration was increased in the SSM population of

CCM hearts when glutamate was used as a substrate, but was not altered with any other substrates (Fig. 4B). RCR was decreased in CCM heart SSM only when glutamate or octanoyl-CoA were used as substrates (Fig. 4C). The ADP/O ratio did not differ in the SSM of CCM hearts with any substrates investigated (Fig. 4D). Consistent with decreased state 3 respiration rates with lipid substrates, a slight, but significant, decrease in MCAD activity was observed in CCM vs. wild-type hearts (Supplemental Table 2).

*Disruption of gene expression oscillations in CCM vs. wild-type hearts.* Although the modest defects in mitochondrial function (e.g., decreased state 3 respiration in CCM heart SSM) may limit energetic reserve at high workloads, they do not readily explain the differences in myocardial metabolism (e.g., increased oleate oxidation rates, decreased lactate release rates) observed between wild-type and CCM hearts. Because the circadian clock is a transcriptionally based mechanism, and the primary intervention in our CCM model is cell-type-specific expression of a dominant-negative transcription factor, diurnal variations in gene expression were next investigated in atria and ventricles isolated from 8-wk-old wild-type and CCM mice, through microarray analyses.

Of the approximate 46,000 genes/transcripts interrogated through microarray analysis, over 15,000 were expressed in

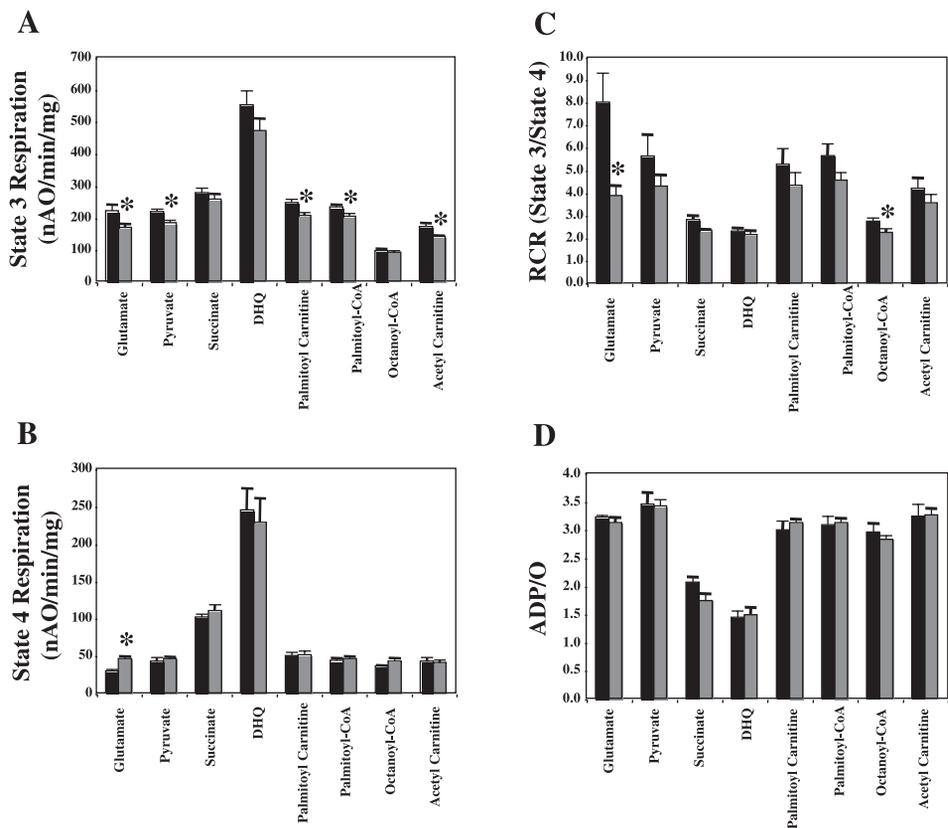
Fig. 3. Diurnal variations in myocardial metabolism of WT and CCM hearts perfused ex vivo at normal (a) and high (b) workloads. Exogenous oleate oxidation rates (A) and endogenously derived lactate release rates (a marker of glycogenolysis; B) were measured in WT and CCM hearts perfused ex vivo at ZT0, ZT6, ZT12, and ZT18, under normal (50-mmHg afterload; a) and high (80-mmHg afterload plus 1  $\mu$ M epinephrine; b) workload conditions. Mice were 11 wk of age. Data are shown as means  $\pm$  SE for between 7 and 9 hearts per group. \* $P$  < 0.05 for post hoc time point comparisons.

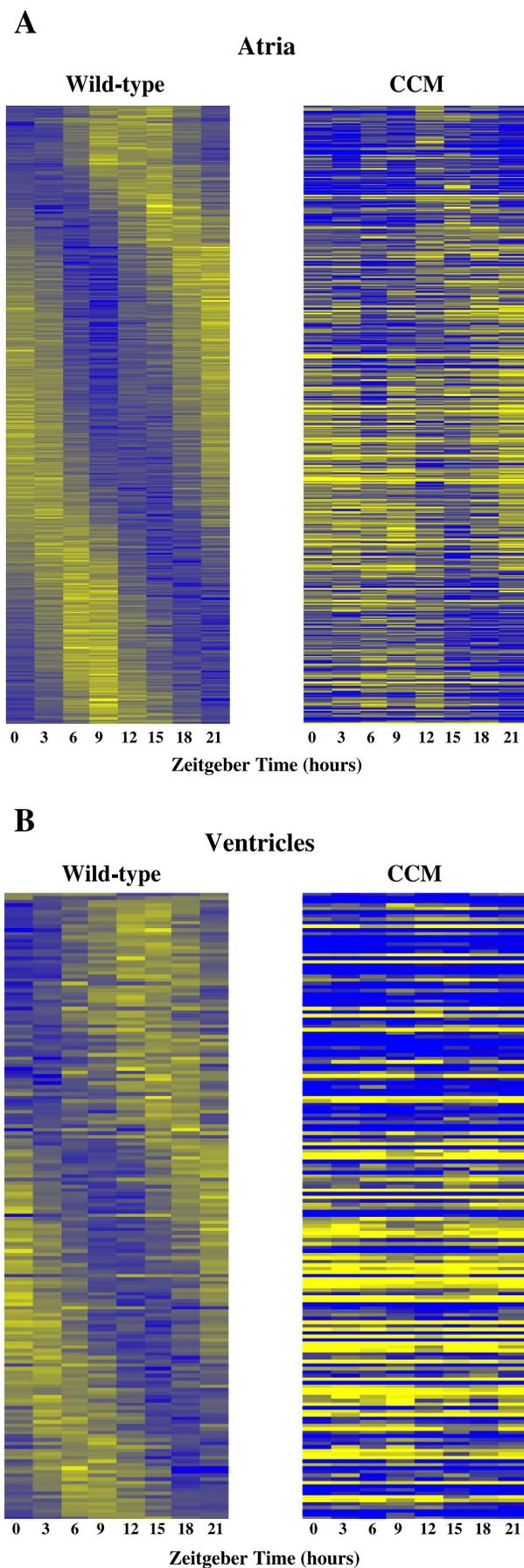


wild-type atria and ventricles, consistent with previous studies (19, 32). Of those genes expressed within wild-type atria, 728 exhibited significant circadian oscillations in expression. This value was markedly lower for wild-type ventricles, wherein only 296 genes exhibit significant circadian oscillations in expression. Of those genes oscillating in wild-type atria and

ventricles, 548 and 176 exhibit significantly altered circadian oscillations in CCM mouse atria and ventricles, respectively; 45 genes were common to both lists. Interestingly, those genes whose expression peaked at the dark-to-light phase transition tended to be constitutively induced in CCM atria and ventricles, whereas those genes whose expression peaked at the

Fig. 4. State 3 respiration (A), state 4 respiration (B), respiratory control ratio (RCR; C), and ratio of oxygen consumed following addition of known amount of ADP (ADP/O ratio; D) for WT vs. CCM subsarcolemmal mitochondria (SSM). SSM were isolated from 11-wk-old mice at ZT18. Data are shown as means  $\pm$  SE for between 5 and 6 hearts per group. DHQ, durohydroquinone. Wild-type, black bars; CCM, gray bars. nAO, Nano atoms of oxygen. \* $P$  < 0.05, WT vs. CCM.





light-to-dark phase transition tended to be constitutively repressed in CCM atria and ventricles (Fig. 5). The complete list of genes is included in Supplemental Table 3.

For validation purposes, a subset of genes identified by the microarray analysis as being altered in CCM hearts was confirmed by quantitative RT-PCR (Supplemental Fig. 5). Eleven genes were chosen to represent a spectrum of amplitudes and phases in gene expression oscillations for wild-type hearts, as well as examples of induction and repression in CCM hearts. In addition, of the 11 genes investigated through RT-PCR analysis, 5 are known core circadian clock components or output genes. These included *bmal1*, *per3*, *dec1*, *e4bp4*, and *dbp*. The microarray analysis identified additional clock component and output genes (e.g., *clock* and *tef*), although RT-PCR was not performed for these. We also confirmed that *adpn*, *dgat2*, *mcip1*, *pbef1*, *pik3r1*, and *prkar1a* exhibit a marked diminution in their circadian oscillation in expression for CCM hearts.

Gene ontology analysis was employed as a means to identify potential biological and molecular functions for the circadian clock within the cardiomyocyte. Figure 6 shows an enrichment of genes in multiple gene ontology categories that would be expected to influence myocardial contractile function and metabolism. These include genes directly involved in transport, transcription, signal transduction, protein turnover, and metabolism. In an attempt to understand further the potential roles of the circadian clock within the cardiomyocyte, Kegg pathway analysis was performed on this novel microarray data set. Supplemental Table 4 shows pathway enrichment for atria and ventricles. Circadian rhythm was identified as a highly enriched pathway in both atria and ventricles, validating the robustness of our array. Consistent with our gene ontology analysis, several signal transduction pathways were identified by the Kegg analysis, including the focal adhesion cascade (Supplemental Table 4). Because many of the identified genes may be influenced by common transcriptional elements, a promoter analysis was performed to identify candidate transcription factors potentially linking the circadian clock to target genes. Supplemental Table 5 shows that multiple common transcriptional elements were identified, including aryl hydrocarbon receptor nuclear translocator and retinoid-related orphan receptor- $\alpha$ , two elements known to be directly regulated by the circadian clock (13, 38).

#### DISCUSSION

The purpose of the present study was to determine whether disruption of the circadian clock within the cardiomyocyte influences myocardial contractile function, metabolism, and/or gene expression, in an attempt to delineate the potential roles of this molecular mechanism in the heart. We find that disruption of the circadian clock within the cardiomyocyte is asso-

Fig. 5. Diurnal variations in expression of genes in atria (A) and ventricles (B) isolated from WT and CCM mice. Blue represents low level of expression; yellow represents high level of expression. "Heat maps" were generated by centering microarray data relative to the mean normalized expression across the time course investigated, followed by scaling each gene, such that the values displayed have unit variance. The relative ordering for the rows was generated according to the phase information determined for the WT data; the same phase ordering was then applied to the CCM data. Mice were 8 wk of age. Data are shown as the mean for 4 separate observations per group.

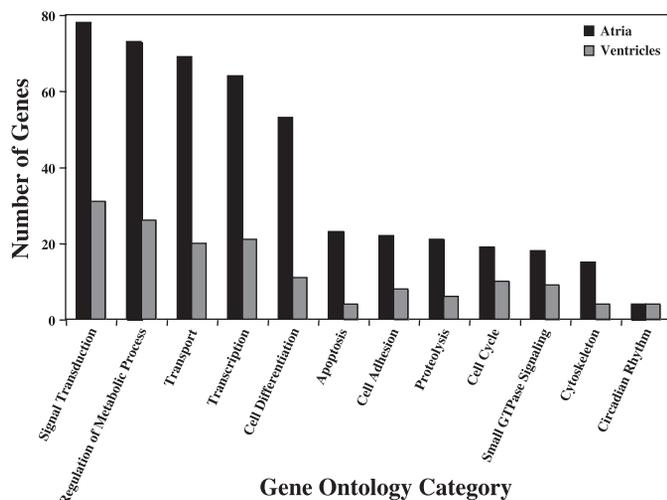


Fig. 6. Gene ontology analysis of genes identified as being potentially regulated by the circadian clock within atrial and ventricular cardiomyocytes.

ciated with 1) decreased diurnal variations in heart rate in vivo; 2) sinus bradycardia both in vivo and ex vivo; 3) loss of diurnal variations in cardiac power ex vivo; 4) increased fatty acid oxidation and decreased cardiac efficiency ex vivo; 5) loss of diurnal variations in the responsiveness of the heart to changes in workload ex vivo; 6) population-specific mitochondrial defects; and 7) alterations in the expression of an array of myocardial genes influencing transport, transcription, signal transduction, protein turnover, and metabolism. These novel observations support the idea that the circadian clock within the cardiomyocyte enables the heart to anticipate circadian rhythms in extracellular stimuli (such as workload), therefore allowing both a rapid and temporally appropriate response.

*The circadian clock within the cardiomyocyte influences cardiovascular function.* Circadian rhythms in myocardial physiology and pathophysiology have classically been explained in terms of extracardiac influences (23, 28, 31, 37). Changes in autonomic, sympathetic, and adrenergic stimulation, nutrients (e.g., glucose, fatty acids, lipoproteins), circulating hormones (e.g., insulin, cortisol, adipokines), as well as vascular resistance and afterload, have all been suggested to influence circadian rhythms in myocardial gene expression, metabolism, and contractile function (42). The present study highlights the existence of an intrinsic molecular mechanism within the cardiomyocyte that likely augments or modulates myocardial biology. Data presented within Figs. 1 and 2A strongly suggest that the circadian clock within the cardiomyocyte influences heart rate. Diurnal variations in heart rate are blunted in CCM mice in vivo (Fig. 1Ac). In addition, CCM hearts exhibit bradycardia both in vivo and ex vivo, in the absence of conduction defects (Figs. 1 and 2A). Taken together, these observations provide strong evidence supporting the hypothesis that the circadian clock within the cardiomyocyte directly regulates heart rate, potentially allowing the heart to anticipate increased activity during the awake (dark) phase. The observation that bradycardia persists for CCM hearts ex vivo, in the absence of acute neurohumoral influences, suggests potential intrinsic regulation of heart rate by the circadian clock within cardiomyocytes of the sinoatrial node.

Diurnal variations in cardiac power persisted in wild-type mouse hearts ex vivo, peaking in the middle of the dark

(awake) phase (Fig. 2Da). These circadian rhythms were absent in CCM hearts, suggesting that the circadian clock within the cardiomyocyte contributes toward increased cardiac output at this time of day. We subsequently hypothesized that the circadian clock within the cardiomyocyte would facilitate a rapid response to increased workload during the waking period. Consistent with this hypothesis, our studies ex vivo (Fig. 2Db) show that wild-type hearts exhibit the greatest responsiveness to an acute increase in workload during the active (dark) phase, in terms of cardiac power. These data provide evidence that increased cardiac output observed in vivo during the dark phase is not solely a function of increased stimulation (e.g., sympathetic and adrenergic stimulation) during the active phase, but that increased intrinsic responsiveness of the myocardium to the stimulus at this time contributes to this phenomenon. The fact that CCM hearts lose diurnal variations in responsiveness to the workload challenge suggests that increased responsiveness of the heart to an episode of elevated workload during the dark phase is mediated by the circadian clock within the cardiomyocyte.

Data presented in Fig. 2 cannot dissociate the relative contributions of adrenergic stimulation and increased afterload on steady-state myocardial function. However, in the present study, hearts were challenged with epinephrine 1 min before the increase in afterload (to mimic the “fight-or-flight” response). For a subset of hearts, developed pressure and heart rate data were collected during this 1-min interval. Data presented within Supplemental Fig. 6 indicate that wild-type hearts exhibit a diurnal variation in their responsiveness to adrenergic stimulation, and that this is mediated by the circadian clock within the cardiomyocyte. Consistent with these observations, multiple G-protein-coupled receptor signaling cascade components were identified by our microarray analysis (e.g., *prkar1a*, the regulatory subunit of PKA, was constitutively elevated in CCM hearts; Supplemental Fig. 5K), raising the possibility that the circadian clock mediates diurnal variations in the sensitivity of the cardiomyocyte to adrenergic stimulation. Given the large number of signaling components altered in CCM hearts, time-of-day-dependent alterations in the phosphorylation status of contractile proteins represent an attractive hypothesis, warranting future investigation.

*The circadian clock within the cardiomyocyte potentially influences myocardial metabolism.* Myocardial contractile function and metabolism are inextricably interlinked. Alterations in contractile function influence myocardial metabolism (and vice versa). Consistent with circadian rhythms in contractile function, our laboratory has previously reported that isolated working rat hearts exhibit marked diurnal variations in both oxidative and nonoxidative metabolism (8, 44). Somewhat surprisingly, wild-type mouse hearts perfused under normal workload conditions did not exhibit diurnal variations in the parameters of myocardial metabolism investigated (Fig. 3 and Supplemental Fig. 2). However, independent of the time of day, CCM hearts perfused under normal workload conditions exhibited increased rates of oxygen consumption and fatty acid oxidation (relative to wild-type hearts; Figs. 2Ea and 3Aa). Consistent with increased rates of myocardial oleate oxidation and oxygen consumption, a number of genes promoting  $\beta$ -oxidation of fatty acids, as well as general mitochondrial oxidative metabolism, were identified as circadian clock-regulated genes that are constitutively induced in CCM atria and ventri-

cles. These include *slc27a1* (fatty acid transport protein 1) and *ndufa3/b3* (a3 and b3 subunits of NADH dehydrogenase) (Supplemental Table 3). However, decreased state 3 respiration in CCM heart SSM (Fig. 4A), as well as decreased MCAD activity in CCM hearts (Supplemental Table 2), suggest that increased rates of oxidative metabolism in perfused CCM hearts may be driven by influences upstream of the electron transport chain. Future studies are required to address this possibility.

During high workload conditions, rates of lactate release (net,  $^{14}\text{C}$ -labeled, and endogenously derived) exhibit a biphasic rhythm in wild-type hearts that is absent in CCM hearts (Fig. 3Bb and Supplemental Fig. 2, *Bii* and *Cii*). In the case of endogenously derived lactate (likely derived from glycogen), we speculate that decreased responsiveness of CCM hearts to adrenergic stimulation may have attenuated glycogen breakdown.

*Disruption of the circadian clock within the cardiomyocyte influences myocardial gene expression.* Consistent with previously published studies, we report that the expression of a large number of genes exhibit significant circadian oscillations in wild-type hearts. For example, Martino et al. (19) reported that 13% of the genes expressed in the mouse heart oscillate in a circadian manner. A similar value (10%) was reported by Storch et al. (32). Consistent with these observations, a higher level of stringency for our microarray analysis (which will eliminate “weaker” oscillations) reveals that ~5% of atrial genes exhibit significant circadian oscillations in expression. Somewhat surprisingly, we find that this value was markedly lower for ventricular genes, being only 2% of expressed genes.

Circadian oscillations in peripheral tissue gene expression are mediated by extracellular (e.g., neurohumoral factors) or intracellular (e.g., circadian clock) influences. Previous studies have investigated circadian oscillations of gene expression in tissues isolated from mice constitutively expressing the CLOCK $\Delta 19$  protein in a ubiquitous manner (i.e., *Clock* mutant mice) (20, 24). However, given the global nature of this mutant model, difficulties arise when addressing the roles of cell-type-specific circadian clocks. Indeed, *Clock* mutant mice exhibit abnormal circadian oscillations in virtually every biological parameter investigated to date, including behavior (sleep-wake cycles, feeding-fasting cycles), neurohumoral factors, as well as organ function and gene expression (24, 36, 40). Using the CCM mouse, in which extracardiac clocks are unaltered (9), the present study was designed to identify genes regulated by the circadian clock within the cardiomyocyte. We find that, of the 728 and 296 genes oscillating in atria and ventricles (respectively) for wild-type mice, 548 and 176 demonstrated significantly altered oscillations in CCM mice, the majority of which are disrupted at the level of amplitude (as opposed to phase; Supplemental Table 3). Gene ontology analysis of this microarray data set suggests that the circadian clock within the cardiomyocyte likely influences a plethora of myocardial functions, including transport, transcription, signal transduction, protein turnover, and metabolism. Clearly, additional studies are required to investigate whether these alterations in genes expression manifest into subsequent alterations in myocardial biology.

*Perspectives, study limitations, and future directions.* One limitation of the present study is the use of a dominant-negative approach to disrupt the circadian clock mechanism. A domi-

nant-negative approach was taken due to marked redundancy of the circadian clock mechanism [e.g., knocking out the *Clock* gene has no effect on the clock mechanism (6)], and that the same dnCLOCK protein is expressed ubiquitously in *Clock* mutant mice (40). However, the possibility remains that the dnCLOCK protein may interfere with CLOCK/brain and muscle ARNT-like-1-independent processes. For this reason, great care has been taken to focus attention on those processes (e.g., contractile function, gene expression, responsiveness to workload challenge) that exhibit a diurnal variation in wild-type, but not CCM, hearts. A second limitation of the model is the constitutive expression of the dnCLOCK protein, which will undoubtedly result in activation of secondary adaptation. For this reason, studies focused on relatively young mice (between 8 and 14 wk of age). An additional potential concern of the present model is that the cardiomyocyte circadian clock may not be completely inactive for CCM hearts; indeed, oscillation of multiple clock components was only attenuated, as opposed to completely abolished, in CCM hearts (Supplemental Fig. 5). However, for these studies, total RNA was isolated from whole ventricles and atria, which are composed of multiple cell types (cardiomyocytes, vascular smooth muscle cells, endothelial cells, fibroblasts), all of which possess cell autonomous clocks. As such, persistence of attenuated circadian clock gene oscillations in CCM hearts not only is expected, but is essential if one is to state that this model is truly cardiomyocyte specific. The present study has investigated diurnal variations (i.e., in the presence of light entrainment), as opposed to classic circadian rhythms (i.e., in the absence of light entrainment). This was to allow interrogation of the potential roles of the circadian clock within the cardiomyocyte under normal physiological conditions. Finally, the present study has not fully characterized the pathophysiological consequences of long-term (i.e., >14 wk) disruption of the circadian clock within the cardiomyocyte on myocardial function. Long-term (i.e., aging) studies, as well as interventions designed to stress CCM hearts (e.g., aortic constriction, myocardial infarctions), are the focus of studies in progress.

*Conclusions.* This is the first study to assess global physiological consequences of disruption of the circadian clock specifically within the cardiomyocyte. We report that, in addition to regulating a plethora of myocardial genes, this transcriptionally based mechanism potentially regulates myocardial contractile function and metabolism. Importantly, we provide evidence that the cardiomyocyte circadian clock influences both heart rate and the responsiveness of the heart to increased workload. As such, both intracellular and extracellular factors should be considered as important mediators of circadian rhythms in cardiovascular function. We speculate that impairment of this molecular mechanism may contribute toward myocardial contractile dysfunction associated with pressure overload, diabetes mellitus, myocardial infarction, and/or shift work (conditions in which the circadian clock within the heart is known to be altered) (9, 18, 43, 45, 46). Future studies should focus on addressing this important hypothesis.

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