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Original article

Neonatal rat cardiomyocytes as an *in vitro* model for circadian rhythms in the heart

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ABSTRACT

Circadian rhythms are biorhythms with a 24-hour period that are regulated by molecular clocks. Several clinical and animal models have been developed to analyze the role of these rhythms in cardiovascular physiology, disease and therapy, but a convenient *in vitro* model that mimics both molecular and functional circadian effects of the heart is not available. Therefore, we established a neonatal rat cardiomyocyte model that recapitulates *in vivo* circadian rhythmicity, as measured by anti-phasic oscillatory mRNA expression of two core clock genes, *Bmal1* and *Per2* and that shows functional dependence on the clock as indicated by an oscillating response in apoptosis induced by doxorubicin, hydroperoxide or hypoxia. In addition, perturbation of the cardiac clock by the use of several compounds including Resveratrol and Ex-527 was found to result in loss of functional rhythmicity. This indicates that neonatal rat cardiomyocytes are a good model to investigate the cardiac circadian clock as well as a system that allows for fast and easy preclinical testing of the influence of compounds on circadian rhythmicity that might have crucial effects on cardiac health.

1. Introduction

Circadian rhythms allow the body to anticipate diurnal environmental changes [1,2]. In humans, these rhythms are regulated by multiple clocks: one central master clock located in the suprachiasmatic nucleus of the brain, and distinct peripheral clocks that are present in almost every organ and cell. The clock pathway relies on the oscillatory expression of core clock genes such as *CLOCK*, *ARNTL*, *PER* and *CRY* [3], which results in the rhythmic expression of clock-controlled output genes (CCGs). According to tissue physiology, CCGs vary per organ and have important functional implications. In the cardiovascular system, circadian rhythms influence various physiological features such as metabolism, electrophysiological characteristics, (cardiac) hormone receptor functionality and coagulation [4–7]. In addition, the clock plays an important role in a number of cardiovascular disorders. The incidence of several diseases, such as myocardial infarction and sudden cardiac death follows a diurnal pattern [8–11], and disruption of rhythmicity by genetic defects, genetic manipulation or sleep disturbance, is involved in cardiac pathophysiology [12–14].

Discovery of the importance of the circadian clock in organ function resulted in several studies that investigated circadian rhythmicity in the heart [13,15,16]. In addition, the interest in the use of circadian rhythms in (pharmacological) therapy is rising [17–19]. Preclinically, several animal models have been applied to uncover the contribution of circadian rhythmicity to cardiac physiology, disease and (pharmacological) therapy [13,20,21]. An easy to use *in vitro* system to analyze cardiac circadian rhythmicity on a transcriptional, protein and functional level, however, is currently lacking.

A handful of experimental studies used adult rodent cardiomyocytes to mimic circadian rhythmicity in the heart [22]. Indeed, *in vitro* cultured rodent ventricular cardiomyocytes retain their circadian rhythms, even in the absence of any environmental input [22]. While these studies have provided important insights, adult cardiomyocytes are difficult to obtain: it takes time to breed animals to adulthood, isolation and culture are difficult and time-consuming, and since adult cardiomyocytes do not proliferate, the number of cells derived per heart is relatively small. In addition, adult cardiomyocytes cannot be kept in culture long enough to allow the analysis of several circadian cycles.

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This limits their suitability for interventional studies, for example to test whether the effect of newly developed drugs is time-dependent and whether they interfere with the intrinsic cardiomyocyte circadian clock.

In the current study, we propose neonatal rat cardiomyocytes (nrCMs) as an easy *in vitro* system to study molecular and functional circadian rhythmicity in the heart and prove that it can serve as a model to test clock interfering characteristics of multiple compounds.

2. Materials and methods

2.1. Isolation of neonatal rat cardiomyocytes

All experiments were carried out in accordance with the Guide for the Care and Use of Laboratory Animals, with prior approval by the Animal Experimentation Ethics Committee, Utrecht University, The Netherlands.

Ventricular cardiomyocytes were isolated from 1-day-old neonatal Wistar rats (Charles River). After sacrifice, hearts were excised and flushed with Solution A (NaCl 8 mg/L, KCl 0,4 mg/L, glucose 1 g/L, $Na_2HPO_4{\cdot}2H_2O$ 60 mg/L, KH_2PO_4 60 mg/L, phenol red 20 mg/L and HEPES 4,77 mg/L in MilliQ pH 7.2–7.4) to get rid of any remaining blood. Atria and large vessels were removed and the ventricles were cut in $1mm^3$ pieces. Tissue pieces were transferred to a glass flask containing 14 mL Solution A supplemented with 750 μL trypsin (2,5%; #15090046, GIBCO) and shaken for 15 min at 37 °C. The tissue/suspension mix was subsequently pipetted up and down several (± 20) times using a glass pipet to detach cells from the tissue pieces. Supernatant was transferred to a new tube, pelleted (3 min at 1100 RPM without brake) and re-suspended in 5 mL culture medium (Ham's F10 without Ca^{2+} and Mg^{2+} (#31550-023, Gibco) supplemented with 1% penicillin-streptomycin (#DE17-602E, Lonza), 1% L-glutamine (BE17-605E, Lonza) and 5% fetal bovine serum (#F7524, Sigma)). New Solution A and trypsin were added to the remaining tissue pieces and the same procedures were followed until no tissue pieces were left (approximately 5 cycles). Cell suspensions were combined, filtered using a sterile non-woven compress (#45847, Cutisoft), and plated on uncoated culture dishes (#430167, Corning). After 2 h, non-adhering cells were collected, counted and plated as a confluent monolayer on laminin-coated (10 mg/L in Solution A, #11243217001, Roche) culture dishes (35 mm, #353001, Falcon). After 20 h, medium was replaced to remove dead cells.

2.2. Bioluminescence reporter recordings

Neonatal rat cardiomyocytes were transduced with Bmal1- and Per2-destabilized luciferase (dLuc) lentiviruses. Lentiviral plasmids, harbouring luciferase reporters of the murine Per2- and Bmal1-promoters, were previously described and kindly provided by Prof. Dr. Liu [23–25]. 1.5 days after transduction, cells were synchronized with 100 nM Dexamethasone for 2 h [26] and switched to recording medium (Phenol Red-free DMEM, 10%FCS, 10 mM HEPES, 0.035% Bicarbonate, 4.5 g/L glucose, Pen/Strep and 100 μM D-Luciferin (Promega). Culture dishes were sealed with high vacuum grease (Dow Corning) and analyzed in a 37 °C incubator using a LumiCycle32 (Actimetrics). Bioluminescence from each dish was continuously recorded (integrated signal of 70 s with intervals of 10 min). Raw data (counts/seconds) were baseline subtracted (polynomial order 3) and smoothened over 1 h.

2.3. Synchronization of nrCMs

nrCMs were synchronized by a 2 h serum shock (SS, 50% culture medium/50% horse serum (#16050-122, Gibco), forskolin (10 μM, #F6886, Sigma) or dexamethasone (100 nM, #D1756, Sigma) for 30 min [26–28]. Non-synchronized cardiomyocytes, that had only a medium change > 1 day before the start of the experiments, served as controls.

2.4. RNA extraction and qRT-PCR

RNA was isolated using phenol-chloroform (Merck) extraction. Purified RNA was treated with DNAse (Promega) and reversibly transcribed with Superscript III reverse transcriptase (ThermoFisher Scientific). mRNA expression was measured using a SYBR Green (Biorad) qRT-PCR. The following primer sequences were used: *Bmal1* (fw): GGCTCATAGATGCAAAAACTGG; *Bmal1* (rv): CTCCAGAACAT AATCGAGATGG). *PPIA* (fw): TTCTGCTGTCTTTGGGACT; *PPIA* (rv): CACCGTGTTCTTCGACATTG.

2.5. Western blotting

For protein analysis, nrCMs were washed with PBS and lysed using RIPA-buffer as described previously [29]. Lysate concentrations were measured using a BCA kit (ThermoFisher Scientific), separated by 10% SDS-PAGE, and transferred to a nitrocellulose membrane. Reverse Ponceau staining was used to quantify protein loading. Membranes were blocked with 5% Protifar (Nutricia), probed with anti-BMAL1 (1:2000, #ab3350, Abcam) antibody, followed by a peroxidase-conjugated antibody (1:7000, #170-6515, Biorad) and ECL chemiluminescence (#sc-2048, SantaCruz) for detection. Ponceau-corrected BMAL1 protein levels were quantified with Image Lab (Version 5.1, Biorad).

2.6. Cell death assay

Induced cell death was quantified using a Caspase-Glo 3/7 (#G8091, Promega) assay and a TUNEL (#11684795910, Roche) assay according to the manufacturer's instructions. nrCMs were isolated, plated (for the Caspase-Glo 3/7 assay in a white clear 96-well plate (#3610, Corning), for the TUNEL assay, on 12 mm glass coverslips in a 24 well plate (#3524, Corning), and synchronized. Between 9 and 51 h (with 6-hour intervals), cells were exposed to several stressors: doxorubicin (10 μM during 6 h, #D1515, Sigma), tert-butyl hydroperoxide (tBHP) 10 μM during 1 h, Sigma) or placing cells in an incubator with 1% O_2 for 3 h (hypoxia), or 3 h followed by 2 h in regular incubator (hypoxia/normoxia). Non-stressed and/or non-synchronized nrCMs served as controls.

2.7. Spontaneous beating

nrCMs were cultured in laminin-coated culture dishes and synchronized. Between 9 and 51 h (with 6-hour intervals), spontaneous beating frequency was measured by manual counting of cardiomyocyte contractions on 6 locations during 1 min (Nixon light microscope, objective 10 ×).

2.8. Compounds

The following concentrations of compounds were used for high and low dose, respectively. Ex-527 (#E7034, Sigma): 250 μM and 25 μM. Resveratrol (#R5010, Sigma): 2.5 μM and 250 nM.

2.9. Statistical analysis

Data are presented as mean ± standard error of mean. Circadian rhythmicity was assessed *via* RAIN, a non-parametric method detecting arbitrary wave-forms in biological data [30]. Student's *t*-test was used to compare non-circadian differences between groups. *P*-values < 0.05 were considered statistically significant.

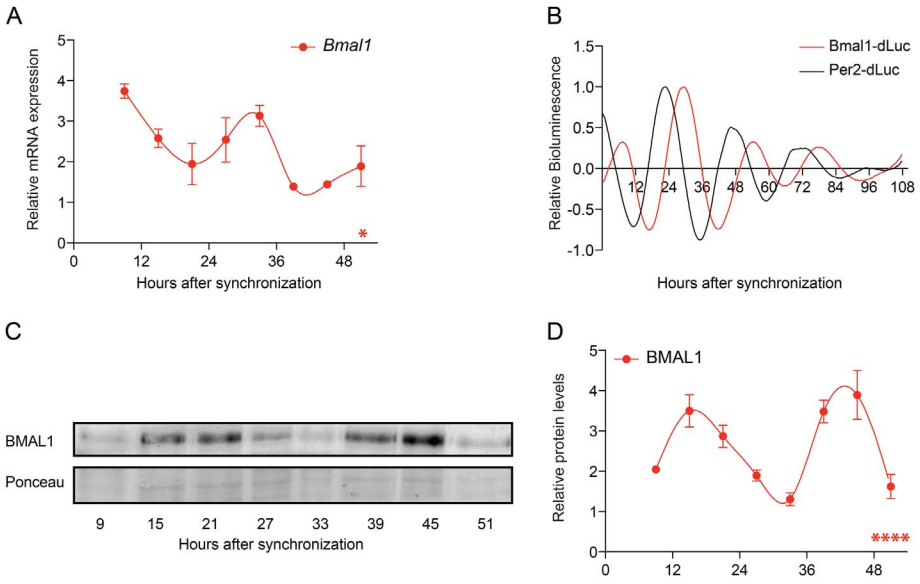

Fig. 1. Neonatal rat cardiomyocytes possess a functional circadian clock. (a) *Bmal1* mRNA expression levels in synchronized neonatal rat cardiomyocytes (nrCMs) as determined by qRT-PCR. Lowest value was set to 1. Expression levels were normalized to the non-oscillatory housekeeping gene *PPIA*. Significance of rhythmicity across 48 h was analyzed using the RAIN algorithm and is indicated (*$P < 0.05$). (b) Bmal1-Luc and Per2-Luc bioluminescence in synchronized nrCMs across 108 h. Data was detrended and normalized to the highest value. Measurements were done using a LumiCyle32. Representative tracks are shown. (c) BMAL1 protein expression as determined through western blot across 48 h. (d) Relative levels protein levels that were deducted from western blots ($n = 3$). Lowest value was set to 1. Ponceau staining was used as a loading control. Significance of rhythmicity across 48 was analyzed using the RAIN algorithm and is indicated (****$P < 0.0005$).

3. Results

3.1. Neonatal rat cardiomyocytes show a functional molecular clock

In contrast to adult cardiomyocytes, neonatal rat cardiomyocytes (nrCMs) are easy to derive in large quantities and can be cultured for prolonged periods. We therefore investigated the use of these cells to study circadian rhythmicity. To analyze the presence of a circadian clock in nrCMs, we synchronized nrCMs *via* serum shock [27]. 24 hour period significance was assessed by the nonparametric algorithm RAIN [30]. *Bmal1* expression, as measured by qRT-PCR, significantly oscillated in a diurnal manner (RAIN, $P < 0.05$; Fig. 1a). To further validate the presence of a functional clock we investigated anti-phasic oscillatory expression of *Bmal1* and *Per2*, a hallmark of a functional circadian clock, using lentiviral promoter-based Bmal1-and Per2-dLuc (destabilized luciferase) reporter constructs. nrCMs were transduced with both constructs, synchronized and bioluminescence was measured using a LumiCycle32. Anti-phasic bioluminescent oscillations for *Bmal1* and *Per2* were detected over the course of 4.5 days (Fig. 1b). In addition to mRNA oscillations, western blot analysis for BMAL1 revealed rhythmic protein levels in nrCMs (RAIN, $P < 0.0005$; Fig. 1c) with peaks that followed gene expression with a delay of approximately 6–12 h (Fig. 1a). From these data, we conclude that nrCMs contain a functional molecular circadian clock at the mRNA as well as at the protein level.

3.2. Neonatal rat cardiomyocytes show 24-hour rhythmicity in beating frequency and induced damage response

A functional circadian clock drives rhythmic output of clock-controlled genes, which allows for functional tissue-specific oscillations. In the heart, central and peripheral circadian clocks together, control heart rate [31]. To test intrinsic functional rhythmicity in nrCMs, we therefore measured beating frequency in a temporal manner. After serum shock-based synchronization, circadian rhythmicity was observed when counting the number of beats per minute over the course of two days (RAIN, $P < 0.05$; Fig. 2a). In contrast, cells that only underwent a medium change 24 h before recording did not show rhythmicity (RAIN, $P = 0.99$; Fig. 2a). These results show that nrCMs do not only possess a molecular clock, but display functional rhythmicity as well.

UBC, a stress-inducible polyubiquitin gene, has previously been shown to oscillate in a diurnal manner in the murine heart and in human ES cell-derived cardiomyocytes [32,33]. In human ES cell-derived cardiomyocytes, this result in a strongly connected oscillatory network of stress-associated transcripts. Therefore, we tested whether synchronized nrCMs display circadian stress-responsive behaviour.

First, we exposed nrCMs to doxorubicin, an anti-cancer drug with known cardiotoxic side effects, in a time-dependent manner. Serum shock based synchronization itself did not induce apoptosis as measured by a Caspase-Glo 3/7 assay (two-tailed Student *t*-test, $P = 0.95$; Supplemental Fig. S1a). When synchronized nrCMs were exposed to doxorubicin however, significant circadian rhythms were found in their damage response, when normalized to background values (Methods) (RAIN, $P < 1E-07$; Fig. 2b; Supplemental Fig. S1b), which were absent in non-synchronized cultures (RAIN, $P = 0.58$; Supplemental Fig. S1c). Additional TUNEL staining on doxorubicin treated nrCMs showed a similar oscillating output (RAIN $P < 0.005$ and $P < 0.0005$ for non-treated and doxorubicin-treated samples; Supplemental Fig. S1d), confirming the time-dependent vulnerability of nrCMs to doxorubicin.

Next, we hypothesized that this time-dependent vulnerability of nrCMs is not limited to doxorubicin and therefore analyzed whether the use of a different stressor, the pro-oxidant tert-butyl hydroperoxide (tBHP) would lead to equal results. Indeed, a circadian apoptotic response pattern was observed for tBHP (RAIN, $P < 0.0005$; Fig. 2c). *In vivo*, the most common cardiac stressors are ischemia and ischemia followed by reperfusion, which are known to follow a diurnal pattern [34]. To mimic ischemia *in vitro*, we cultured nrCMs in hypoxic conditions (1% O_2) during 3 h at a 6-hour interval. To mimic ischemia/reperfusion, 3-hour hypoxia conditions were followed by 2 h of normoxia (5% CO_2 and 20% O_2). The apoptotic response of nrCMs after hypoxia treatment as well as hypoxia/normoxia followed a significant 24-hour pattern (RAIN $P < 0.005$ and $P < 0.05$, respectively; Fig. 2d). These results show that, similar to *in vivo* hearts, nrCMs respond to ischemia in a circadian manner, which further underscores the relevance of using nrCMs when aiming to assess the role of rhythmicity in response to stressors.

3.3. Neonatal rat cardiomyocytes function as a good model for molecular cardiac clock interference in vitro

Many pharmacological drugs and compounds affect the circadian clock. Because of the important role of circadian rhythmicity in cardiovascular physiology and disease, an *in vitro* tool that is able to predict the effect of compounds on the molecular and functional clock could be useful for drug development and testing. To determine whether nrCMs can be used for this purpose, we treated nrCMs with resveratrol, a

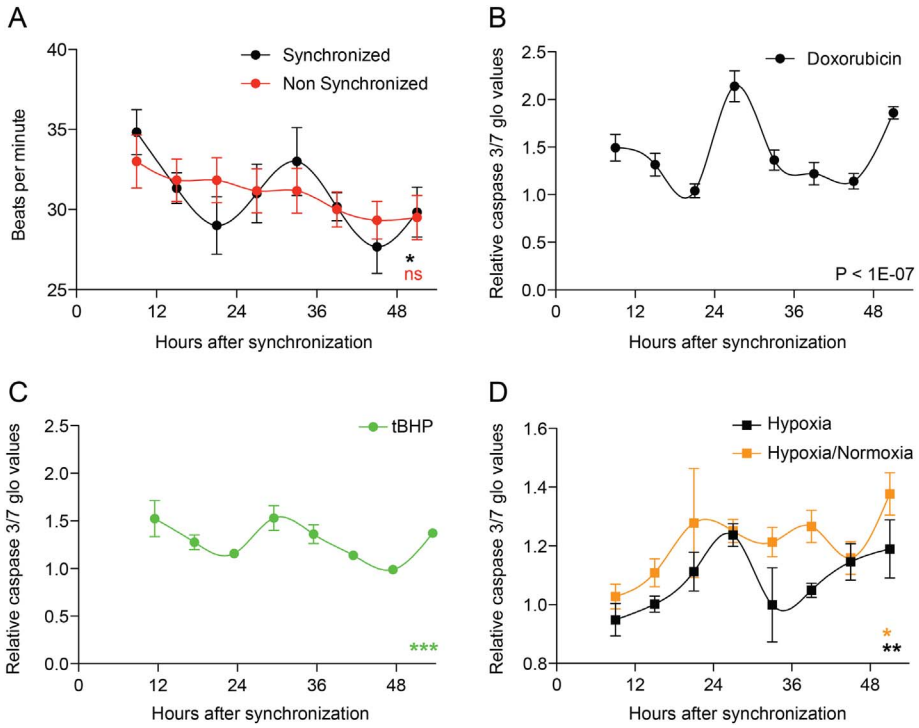

Fig. 2. Neonatal rat cardiomyocytes show functional oscillations. (a) Counts of spontaneous contraction in nrCMs, measured as beats per minute and monitored across 48 h ($n = 6$). (b) Relative apoptotic levels in doxorubicin treated synchronized nrCMs measured by Caspase-Glo 3/7 values across 48 h. Values are normalized to non-treated cardiomyocytes. (c) Similar analysis as in b) for tBHP. (d) Similar analysis as b) for hypoxia or hypoxia followed by normoxia treatment across 48 h. Significance of rhythmicity in (a–d) across 48 was analyzed using the RAIN algorithm and is indicated (ns: not significant, $*P < 0.05$, $**P < 0.005$, $***P < 0.0005$).

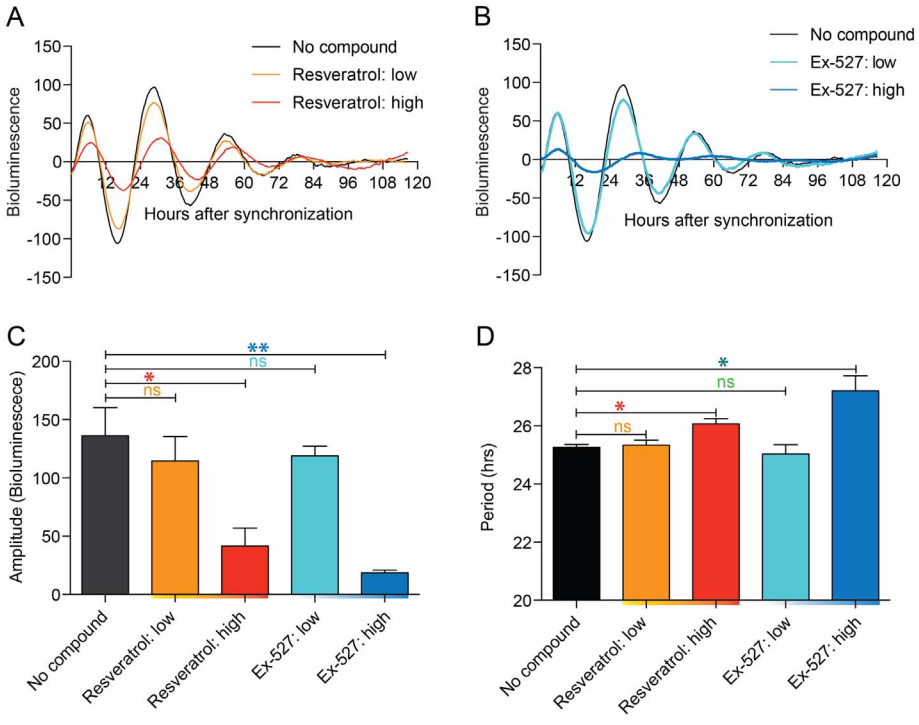

Fig. 3. Resveratrol and Ex-527 affect the molecular clock of neonatal rat cardiomyocytes. (a) Per2-Luc bioluminescence in synchronized neonatal rat cardiomyocytes (nrCMs) treated with low or high doses of resveratrol. Bioluminescence was measured by a LumiCyle32 across 116 h. Means of 3 independent replicates are shown. (b) Similar analysis as in a) for nrCMs treated with low or high doses of Ex-527. (c) Per2-dLuc amplitudes in nrCMs treated with resveratrol or Ex-527. (d) Period of circadian waves in nrCMs treated with resveratrol or Ex-527. Difference between conditions in c) and d) were analyzed using an unpaired Student's *t*-test and significance levels are indicated (ns: not significant, $*P < 0.05$, $**P < 0.005$).

compound of interest for its potentially beneficial effects on atherosclerosis, hypertension and ischemia/reperfusion [35], which has also been linked to the circadian clock [36,37]. Using our Per2-dLuc bioluminescence reporter system, we observed a dose-dependent decrease of Per2 amplitude (Fig. 3a), which indicates dampening of the molecular clock upon administration of resveratrol. Congruently, we observed a dose-dependent dampening of circadian oscillations upon administration of Ex-527 (Sirtinol) (Fig. 3b), a compound previously linked to both cardiomyocyte stress [38], and the core clock system *via* the sirtuin SIRT1 [39]. Low concentrations of each compound (resveratrol or Ex-527) had moderate effects, but importantly, high dosage significantly decreased the amplitude of Per2-dLuc levels (Student's *t*-test: resveratrol, $P < 0.05$; Ex-527, $P < 0.005$; Fig. 3c). Additionally, both compounds lengthened the circadian period at high dose (Student's *t*-test $P < 0.05$ for resveratrol, and $P < 0.05$ for Ex-527 respectively). Dampened circadian amplitudes have been correlated with premature aging [40] and clock disturbance has been observed in numerous pathologies (hypertension, diabetes, sleep disturbance and cancer [1,14]). Gaining insight in the consequences of compounds on the cardiac clock is thus essential considering the pros and cons of using

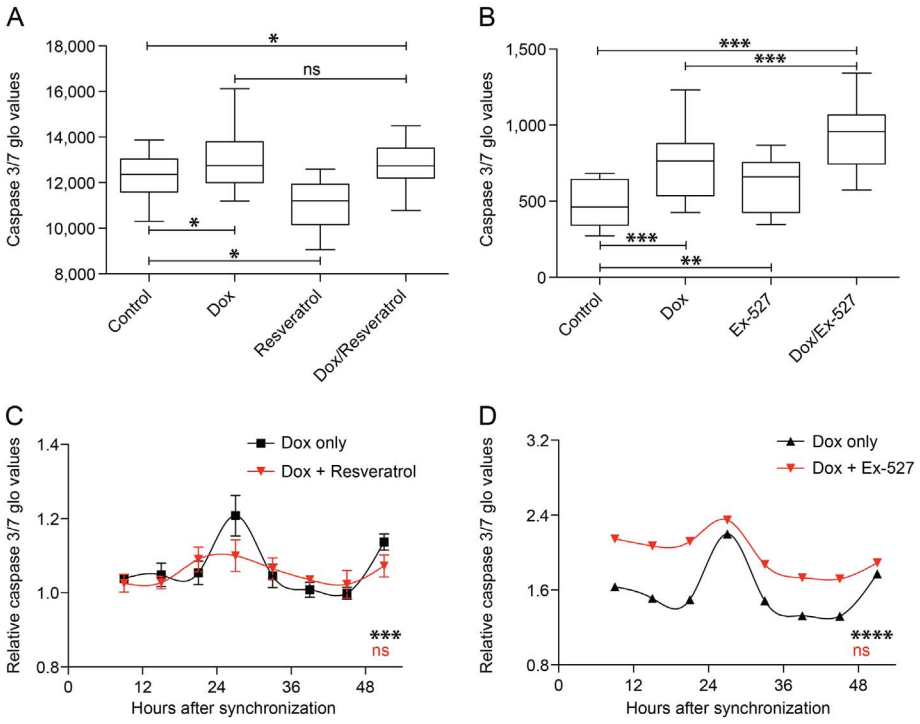

Fig. 4. Resveratrol and Ex-527 impair time-dependent apoptotic response to doxorubicin in neonatal rat cardiomyocytes. (a) Apoptotic levels in neonatal rat cardiomyocytes (nrCMs) treated with doxorubicin (dox), resveratrol or a combination compared to non-treated cells (control). (b) Similar analysis as in a) for Ex-527 treatment. (c) Relative doxorubicin-induced apoptosis levels in nrCMs treated with resveratrol across 48 h. Apoptotic levels were normalized to baseline apoptosis levels of non-treated cells. (d) Similar analysis as in c) for Ex-527 treatment across 48 h. Effect between groups in a) and b) were analyzed using unpaired Student's *t*-test (ns: not significant, $^{*}P < 0.05$, $^{**}P < 0.005$, $^{***}P < 0.0005$). Significance of rhythmicity in c) and d) were analyzed using RAIN and indicated ($^{**}P < 0.005$, $^{****}P < 0.00005$).

a specific compound. As illustrated here, nrCMs would be an excellent system to model how pharmacological therapeutics affect clock dynamics.

3.4. Neonatal rat cardiomyocytes lose functional rhythmicity upon clock disturbance

As both resveratrol and Ex-527 were found to dampen the molecular clock in nrCMs (Fig. 3a–d), we questioned whether this would also affect functional circadian output. Resveratrol showed a cardioprotective effect on nrCMs under basic culture conditions (Student's *t*-test $P < 0.05$; Fig. 4a). Nevertheless, the strong pro-apoptotic effect of doxorubicin (Student's *t*-test $P < 0.05$) could not be reversed by resveratrol (Fig. 4a). In contrast, when our second compound Ex-527 was added, basal apoptosis levels increased significantly (Student's *t*-test $P < 0.005$; Fig. 4B). Administration of Ex-527 in combination with doxorubicin had an additive apoptotic effect when compared to doxorubicin alone (Student's *t*-test, $P < 0.0005$; Fig. 4b).

Analysis of the effect of both compounds on the oscillating response to doxorubicin (Fig. 2c) revealed that both resveratrol and Ex-527 abolished the circadian variation of the apoptotic response (RAIN, resveratrol, $P = 0.29$, Ex-527, $P < 0.37$; Fig. 4c,d). This shows that disturbing the molecular clock of nrCMs through the use of compounds can lead to impaired circadian functionality.

4. Discussion

Circadian rhythmicity plays an important role in physiological, biochemical, and behavioral processes and is increasingly considered in pharmacological studies [19]. Typically, pharmacological studies are done in animal models, where time-of-day dependent drug efficacy or toxicity has to be analyzed at various time-points, requiring an undesired, but necessary large number of animals. In addition, there has been a shift in attention from central, neurohumoral control of 24-h rhythms to peripheral, cellular regulation [13]. In animals experiments, these different mechanisms cannot be uncoupled completely, possibly blurring circadian effects. Therefore, alternative, more specific assays at earlier preclinical test stages are needed. In cardiovascular disease this is challenging because cardiomyocytes are difficult to obtain or to maintain in culture for a sufficient long time without deterioration of intrinsic characteristics.

In the current study, we present neonatal rat cardiomyocytes (nrCMs) as a convenient system to model the circadian clock of the heart. We show that nrCMs have a molecular and functional clock with 24-hour rhythmicity in spontaneous contraction as well as resistance to induced apoptosis by several stressors. Importantly, we demonstrate that different pharmacological compounds influence and disrupt the cardiac clock both at the molecular and functional level.

Our findings correspond to *in* and *ex vivo* studies that investigated circadian rhythmicity in the heart. Clock components (BMAL1 and PER2), stress tolerance, and contraction rate are regulated by the cardiomyocyte circadian clock and all follow a 24-hour pattern *in vivo* [4,31]. More specifically, in a rodent model Durgan et al. demonstrated that cardiomyocyte stress tolerance is lowest when *Bmal1* mRNA expression levels start to rise [34]. Our *in vitro* assay shows similar results: maximum apoptosis (doxorubicin exposure 20–26 h after synchronization) coincided with low, increasing *Bmal1* mRNA levels. Second, *in vitro* circadian spontaneous contraction rhythmicity is comparable to physiological rhythmicity: heart rate in mice (both *in* and *ex vivo*) peaks in the middle of the active period, when *Bmal1* mRNA expression is high [20]. In correspondence, our nrCM experiments show maximal spontaneous beating frequencies when *Bmal1* mRNA is high.

Prior to using expensive and long-lasting animal or clinical studies, nrCMs would thus be a good and easy-to-use first stage model to study the cardiomyocyte molecular clock *in vitro*, as well as the effect of compounds on circadian rhythmicity that might have crucial effects on cardiac health.

Disclosures

None.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.yjmcc.2017.08.009.

References

[1] P. Dierickx, B. Du Pré, D.A.M. Feyen, N. Geijsen, T. van Veen, P.A. Doevendans, et al., Circadian Rhythms in Stem Cell Biology and Function, in: Stem Cells and Cardiac Regeneration, Springer International Publishing, Cham, 2015, pp. 57–78, http://dx.doi.org/10.1007/978-3-319-25427-2_5.

[2] B.C. Du Pré, T.A.B. van Veen, M.E. Young, M.A. Vos, P.A. Doevendans, L.W. van Laake, Circadian rhythms in cell maturation, Physiology 29 (2014) 72–83, http://dx.doi.org/10.1152/physiol.00036.2013.

[3] J.S. Takahashi, Molecular components of the circadian clock in mammals, Diabetes Obes. Metab. 17 (Suppl. 1) (2015) 6–11, http://dx.doi.org/10.1111/dom.12514.

[4] D.J. Durgan, M.E. Young, The cardiomyocyte circadian clock: emerging roles in health and disease, Circ. Res. 106 (2010) 647–658, http://dx.doi.org/10.1161/CIRCRESAHA.109.209957.

[5] F.A.J.L. Scheer, A.D. Michelson, A.L. Frelinger, H. Evoniuk, E.E. Kelly, M. McCarthy, et al., The human endogenous circadian system causes greatest platelet activation during the biological morning independent of behaviors, PLoS One 6 (2011) e24549, , http://dx.doi.org/10.1371/journal.pone.0024549.

[6] K. Witte, R. Parsa-Parsi, M. Vobig, B. LEMMER, Mechanisms of the circadian regulation of beta-adrenoceptor density and adenylyl cyclase activity in cardiac tissue from normotensive and spontaneously hypertensive rats, J. Mol. Cell. Cardiol. 27 (1995) 1195–1202.

[7] D. Jeyaraj, S.M. Haldar, X. Wan, M.D. McCauley, J.A. Ripperger, K. Hu, et al., Circadian rhythms govern cardiac repolarization and arrhythmogenesis, Nature 483 (2012) 96–99, http://dx.doi.org/10.1038/nature10852.

[8] M. Peckova, C.E. Fahrenbruch, L.A. Cobb, A.P. Hallstrom, Circadian variations in the occurrence of cardiac arrests: initial and repeat episodes, Circulation 98 (1998) 31–39.

[9] J.E. Muller, P.H. Stone, Z.G. Turi, J.D. Rutherford, C.A. Czeisler, C. Parker, et al., Circadian variation in the frequency of onset of acute myocardial infarction, N. Engl. J. Med. 313 (1985) 1315–1322.

[10] J.E. Muller, G.H. Tofler, P.H. Stone, Circadian variation and triggers of onset of acute cardiovascular disease, Circulation 79 (1989) 733–743.

[11] F. Portaluppi, R.C. Hermida, Circadian rhythms in cardiac arrhythmias and opportunities for their chronotherapy, Adv. Drug Deliv. Rev. 59 (2007) 940–951, http://dx.doi.org/10.1016/j.addr.2006.10.011.

[12] M.V. Vyas, A.X. Garg, A.V. Iansavichus, J. Costella, A. Donner, L.E. Laugsand, et al., Shift work and vascular events: systematic review and meta-analysis, BMJ 345 (2012) e4800, , http://dx.doi.org/10.1136/bmj.e4800.

[13] T.A. Martino, M.E. Young, Influence of the cardiomyocyte circadian clock on cardiac physiology and pathophysiology, J. Biol. Rhythm. (2015), http://dx.doi.org/10.1177/0748730415575246.

[14] P.Y. Woon, P.J. Kaisaki, J. Bragança, M.-T. Bihoreau, J.C. Levy, M. Farrall, et al., Aryl hydrocarbon receptor nuclear translocator-like (BMAL1) is associated with susceptibility to hypertension and type 2 diabetes, Proc. Natl. Acad. Sci. U. S. A. 104 (2007) 14412–14417, http://dx.doi.org/10.1073/pnas.0703247104.

[15] M.E. Young, Temporal partitioning of cardiac metabolism by the cardiomyocyte circadian clock, Exp. Physiol. 101 (2016) 1035–1039, http://dx.doi.org/10.1113/EP085779.

[16] S. Beesley, T. Noguchi, D.K. Welsh, Cardiomyocyte circadian oscillations are cell-autonomous, amplified by β-adrenergic signaling, and synchronized in cardiac ventricle tissue, PLoS One 11 (2016) e0159618, , http://dx.doi.org/10.1371/journal.pone.0159618.

[17] E. Ortiz-Tudela, A. Mteyrek, A. Ballesta, P.F. Innominato, F. Lévi, Cancer chronotherapeutics: experimental, theoretical, and clinical aspects, Handb. Exp. Pharmacol. 217 (2013) 261–288, http://dx.doi.org/10.1007/978-3-642-25950-0_11.

[18] R.C. Hermida, D.E. Ayala, A. Mojón, J.R. Fernández, Decreasing sleep-time blood pressure determined by ambulatory monitoring reduces cardiovascular risk, J. Am. Coll. Cardiol. 58 (2011) 1165–1173, http://dx.doi.org/10.1016/j.jacc.2011.04.043.

[19] S. Ohdo, Chronotherapeutic strategy: rhythm monitoring, manipulation and disruption, Adv. Drug Deliv. Rev. 62 (2010) 859–875, http://dx.doi.org/10.1016/j.addr.2010.01.006.

[20] M.S. Bray, C.A. Shaw, M.W.S. Moore, R.A.P. Garcia, M.M. Zanquetta, D.J. Durgan, et al., Disruption of the circadian clock within the cardiomyocyte influences myocardial contractile function, metabolism, and gene expression, Am. J. Physiol. Heart. C 294 (2008) H1036–47, http://dx.doi.org/10.1152/ajpheart.01291.2007.

[21] D.J. Durgan, J.-Y. Tsai, M.H. Grenett, B.M. Pat, W.F. Ratcliffe, C. Villegas-Montoya, et al., Evidence suggesting that the cardiomyocyte circadian clock modulates responsiveness of the heart to hypertrophic stimuli in mice, Chronobiol. Int. 28 (2011) 187–203, http://dx.doi.org/10.3109/07420528.2010.550406.

[22] R.A. Peliciari-Garcia, M.M. Zanquetta, J. Andrade-Silva, D.A. Gomes, M.L. Barreto-Chaves, J. Cipolla-Neto, Expression of circadian clock and melatonin receptors within cultured rat cardiomyocytes, Chronobiol. Int. 28 (2011) 21–30, http://dx.doi.org/10.3109/07420528.2010.525675.

[23] A.C. Liu, D.K. Welsh, C.H. Ko, H.G. Tran, E.E. Zhang, A.A. Priest, et al., Intercellular coupling confers robustness against mutations in the SCN circadian clock network, Cell 129 (2007) 605–616, http://dx.doi.org/10.1016/j.cell.2007.02.047.

[24] A.C. Liu, H.G. Tran, E.E. Zhang, A.A. Priest, D.K. Welsh, S.A. Kay, Redundant function of REV-ERBα and β and non-essential role for Bmal1 cycling in transcriptional regulation of intracellular circadian rhythms, PLoS Genet. 4 (2008) e1000023, , http://dx.doi.org/10.1371/journal.pgen.1000023.

[25] C. Ramanathan, S.K. Khan, N.D. Kathale, H. Xu, A.C. Liu, Monitoring cell-autonomous circadian clock rhythms of gene expression using luciferase bioluminescence reporters, J. Vis. Exp. (2012), http://dx.doi.org/10.3791/4234.

[26] A. Balsalobre, S.A. Brown, L. Marcacci, F. Tronche, C. Kellendonk, H.M. Reichardt, et al., Resetting of circadian time in peripheral tissues by glucocorticoid signaling, Science 289 (2000) 2344–2347.

[27] A. Balsalobre, F. Damiola, U. Schibler, A serum shock induces circadian gene expression in mammalian tissue culture cells, Cell 93 (1998) 929–937.

[28] K. Yagita, H. Okamura, Forskolin induces circadian gene expression of rPer1, rPer2and dbpin mammalian rat-1 fibroblasts, FEBS Lett. 465 (1999) 79–82, http://dx.doi.org/10.1016/S0014-5793(99)01724-X.

[29] T.A.B. van Veen, H.V.M. van Rijen, R.F. Wiegerinck, T. Opthof, M.C. Colbert, S. Clement, et al., Remodeling of gap junctions in mouse hearts hypertrophied by forced retinoic acid signaling, J. Mol. Cell. Cardiol. 34 (2002) 1411–1423.

[30] P.F. Thaben, P.O. Westermark, Detecting rhythms in time series with RAIN, J. Biol. Rhythm. 29 (2014) 391–400, http://dx.doi.org/10.1177/0748730414553029.

[31] Y. Yaniv, E.G. Lakatta, The end effector of circadian heart rate variation: the sinoatrial node pacemaker cell, BMB Rep. 48 (2015) 677–684.

[32] R. Zhang, N.F. Lahens, H.I. Ballance, M.E. Hughes, J.B. Hogenesch, A circadian gene expression atlas in mammals: implications for biology and medicine, Proc. Natl. Acad. Sci. U. S. A. 111 (2014) 16219–16224, http://dx.doi.org/10.1073/pnas.1408886111.

[33] P. Dierickx, M.W. Vermunt, M.J. Muraro, M.P. Creyghton, P.A. Doevendans, A. van Oudenaarden, et al., Circadian networks in human embryonic stem cell-derived cardiomyocytes, EMBO Rep. (2017), http://dx.doi.org/10.15252/embr.201743897.

[34] D.J. Durgan, T. Pulinilkunnil, C. Villegas-Montoya, M.E. Garvey, N.G. Frangogiannis, L.H. Michael, et al., Short communication: ischemia/reperfusion tolerance is time-of-day-dependent: mediation by the cardiomyocyte circadian clock, Circ. Res. 106 (2010) 546–550, http://dx.doi.org/10.1161/CIRCRESAHA.109.209346.

[35] G. Petrovski, N. Gurusamy, D.K. Das, Resveratrol in cardiovascular health and disease, Ann. N. Y. Acad. Sci. 1215 (2011) 22–33, http://dx.doi.org/10.1111/j.1749-6632.2010.05843.x.

[36] H. Oike, M. Kobori, Resveratrol regulates circadian clock genes in rat-1 fibroblast cells, bioscience, biotechnology, and, Biochemistry 72 (2014) 3038–3040, http://dx.doi.org/10.1271/bbb.80426.

[37] L. Sun, Y. Wang, Y. Song, X.-R. Cheng, S. Xia, M.R.T. Rahman, et al., Resveratrol restores the circadian rhythmic disorder of lipid metabolism induced by high-fat diet in mice, Biochem. Biophys. Res. Commun. 458 (2015) 86–91, http://dx.doi.org/10.1016/j.bbrc.2015.01.072.

[38] M. Gertz, F. Fischer, G.T.T. Nguyen, M. Lakshminarasimhan, M. Schutkowski, M. Weyand, et al., Ex-527 inhibits Sirtuins by exploiting their unique NAD + -dependent deacetylation mechanism, Proc. Natl. Acad. Sci. U. S. A. 110 (2013) E2772–81, , http://dx.doi.org/10.1073/pnas.1303628110.

[39] W. Zheng, Y.-B. Lu, S.-T. Liang, Q.-J. Zhang, J. Xu, Z.-G. She, et al., SIRT1 mediates the protective function of Nkx2.5 during stress in cardiomyocytes, Basic Res. Cardiol. 108 (2013) 364, http://dx.doi.org/10.1007/s00395-013-0364-y.

[40] H.-C. Chang, L. Guarente, SIRT1 mediates central circadian control in the SCN by a mechanism that decays with aging, Cell 153 (2013) 1448–1460, http://dx.doi.org/10.1016/j.cell.2013.05.027.