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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.