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Repercussions of hypo and hyperthyroidism on the heart circadian clock

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Abstract

Myocardial gene expression and metabolism fluctuate over the course of the day in association with changes in energy supply and demand. Time-of-day-dependent oscillations in myocardial processes have been linked to the intrinsic cardiomyocyte circadian clock. Triiodothyronine (T3) is an important modulator of heart metabolism and function. Recently, our group has reported time-of-day-dependent rhythms in cardiac T3 sensitivity, as well as, T3-mediated acute alterations on core clock components. Hypo and hyperthyroidism are the second most prevalent endocrine disease worldwide. Considering the importance of the cardiomyocyte circadian clock and T3 to cardiac physiology, the aim of this study was to investigate the consequences of chronic hypo and hyperthyroidism on 24-h rhythms of circadian clock genes in the heart. Hypo and hyperthyroidism was induced in rats by thyroidectomy (Tx) and i.p. injections of supraphysiological dose of T3, respectively. Here we report alterations in mRNA levels of the major core clock components (*Bmal1*, *Per2*, *Nr1d1* and *Rora*) for both experimental conditions (with the exception of *Per2* during hyperthyroid condition). Oscillations in mRNA levels of key glucose and fatty acid metabolism genes known to be clock controlled (*Pdk4*, *Ucp3*, *Acot1* and *Cd36*) were equally affected by the experimental conditions, especially during the hypothyroid state. These findings suggest that chronic alterations in thyroid status significantly impacts 24-h rhythms in circadian clock and metabolic genes in the heart. Whether these perturbations contribute towards the pathogenesis of cardiac dysfunction associated with hypo and hyperthyroidism requires further elucidation.

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DISCLOSURES

None declared.

Keywords

hypothyroidism; hyperthyroidism; circadian clocks; gene expression; heart; metabolism

INTRODUCTION

Thyroid pathological conditions such as hyper and hypothyroidism are the 2nd most prevalent endocrinopathy in the world, and are considered a significant risk factor for cardiovascular disease (Biondi & Klein, 2004; Jabbar, Pingitore et al., 2017), which in turn, is classified as a major cause of morbidity and mortality in the United States (Mozaffarian, Benjamin et al., 2015; Benjamin, Blaha et al., 2017). According to the American Thyroid Association (ATA), 12 percent of the American population will develop a thyroid disorder throughout life (ATA) and about 20 million Americans already have some form of thyroid disease.

Thyroxine (T₄) is the major product of thyroid gland, while triiodothyronine (T₃) has higher affinity to thyroid hormone receptors (THR) and is mainly derived from T₄ peripheral conversion by deiodinases 1 and 2 (DIO1 and DIO2, respectively) (Maia, Kim et al., 2005). T₃ presents a rhythmic pattern in its biosynthesis processes, and circadian and seasonal oscillations of T₃ levels have been reported (Jordan, Rousset et al., 1980). The daily profile of thyroid hormone secretion seemed to be induced by the rhythmic TSH secretion (Fahrenkrug, Georg et al., 2017) and the acrophase of circulating T₃ levels occurs in the second half of the light phase (Ahlersova, Ahlers et al., 1997; Guo, Liu et al., 2015), while the thyroid hormones concentration in peripheral tissues is modulated by the activity of deiodinases, which are under circadian clock control (Campos-Barros, Musa et al., 1997; Yasuo, Watanabe et al., 2006).

The heart is an important target of T₃ (Klein & Ojamaa, 2001; Danzi & Klein, 2012). It induces the transcription rate of many genes, consequently altering metabolism and contractile function. For example, T₃ modulates expression/activity of MYH6 and MYH7, ATPase Sarcoplasmic/Endoplasmic Reticulum Ca²⁺ Transporting 2 (ATPT2) (Nunes, Bianco et al., 1985; Rohrer & Dillmann, 1988; Biondi, Palmieri et al., 2002; Dillmann, 2002) and its inhibitor, the cardiac phospholamban (PLB) (MacLennan & Kranias, 2003), which in turn influences heart rate, as well as force generation (Morkin, Flink et al., 1983; Dillmann, 1996).

The importance of the heart circadian clock has been extensively investigated over the last decade (Young, Razeghi et al., 2001b; Young, 2006; Durgan & Young, 2010). It has been shown that its functionality is essential for daily fluctuations in myocardial contractile function, metabolism and gene expression (Bray, Shaw et al., 2008), as well as responsiveness of the heart to fatty acids and pro-hypertrophic stimuli (Durgan, Tsai et al., 2011).

The circadian clock consists of a set of proteins that present an auto-regulation by feedback loops with a periodicity of approximately 24 h. Two major components, CLOCK and BMAL1, form a heterodimer that binds to E-boxes near to promoters of many target genes,

including core clock components as Period (*Per*)1,2, and Cryptochrome (*Cry*)1,2, as well as clock-controlled genes (CCG)(Takahashi, 2015). ROR α is also a classic modulator of circadian clock expression, as described for *Bmal1*(Emery & Reppert, 2004), which in turn, induces transcription of other circadian genes and clock-controlled genes, including metabolic genes(Kovac, Husse et al., 2009). Generally, the circadian clock confers anticipation and facilitates the responsiveness pattern of an organ or cell to an external stimuli/stress in an appropriated manner regarding the time of its occurrence.

Recently, our group has demonstrated that the heart shows a greater transcriptional sensitivity to T3 at the end of the dark phase, with significant alterations of mRNA expression of core clock components; showing therefore, a time-of-day-dependent sensitivity to T3 and modulation of the heart circadian clock(Peliciari-Garcia, Previde et al., 2016).

Taken together, this data supports the evidence for T3-mediated modulation of clock and metabolic genes mRNA expression in the heart. Additionally, considering the circadian clock and T3 relevance to cardiac physiology, as well as, the higher incidence of thyroid dysfunctions, the investigation of this relationship became the major aim of this study.

MATERIAL AND METHODS

Animals

Rat. Male Wistar rats weighing 150–180 g were obtained from the Institute of Biomedical Sciences, University of São Paulo, São Paulo, Brazil. The animals were kept on a 12 h/12 h light/dark (LD) cycle (light phase: lights on at 06:00 am = Zeitgeber Time – ZT 0; dark phase: red filter Kodak 1A, 0.5 to 1 lux), in a temperature-controlled room and had access to food and water *ad libitum*. Ethics approval was granted by the Committee of Ethics in Animal Experimentation of the Institute of Biomedical Sciences, University of São Paulo (#81, 21–03).

Experimental design

Two experimental protocols were performed in this study. Hypothyroidism was induced by thyroidectomy followed by 2-Mercapto-1-methylimidazole (MMI [0.03%] plus CaCl₂ [0.05%] in drinking water for 20 days), as described previously(Bargi-Souza, Romano et al., 2013; Romano, Bargi-Souza et al., 2013). Control animals underwent a sham operation, without MMI treatment. Hyperthyroidism was induced in a second set of animals by intraperitoneal (i.p.) injections of T3 [0.75 µg/100 g], twice a day, at ZTs 2 and 14, respectively, for 5 consecutive days(Bargi-Souza, Romano et al., 2013). Saline injections were performed in Control animals. The time of i.p. injections were based on our previous time-of-day dependent T3 sensitivity study demonstrating greatest sensitivity of the heart at ZT14; ZT2 was chosen, as this is exactly 12-h from ZT14. At the end of each protocol, rats were euthanized at one of eight distinct ZTs, heart ventricles were collected rapidly, frozen immediately in liquid N₂, and stored at –80 °C prior to subsequent analysis. At least five animals were included at each ZT per experimental group for all protocols in this study.

Quantitative RT-PCR

Total RNA was extracted from heart ventricles using Trizol, according to the manufacture's instruction. The total RNA quantification was evaluated by spectrophotometry using the Gen 1.10 software and Epoch™ Microplate Spectrophotometer coupled to Take3™ Multi-Volume Plate (BioTek, Winooski, VT, USA). The RNA concentration was obtained from the 260 nm absorbance values using the 40 µg / mL as reference for optical densitometry unit. The ratio between 260/280 nm absorbance was used for purity RNA analysis evaluation. Afterwards, 1 µg of total RNA was plotted into a 1 % agarose gel to verify the integrity of RNA through the visualization of 28S and 18S RNA ribosomal bands. ssDNA synthesis was performed by the use of MMLV-RT (Promega) from 1 µg of total RNA. Candidate gene expression analysis was performed by quantitative RT-PCR, using SYBR GREEN (Invitrogen) as the fluorescent dye, and the 2(-Delta CT) method(Livak & Schmittgen, 2001); the Ct value is the calculated cycle number where the fluorescence signal was emitted significantly above background levels. The primers for *Adrb1*, *Acot1*, *Cd36* and *Myh7* were designed from rat sequences available in GenBank (Table 1); other primer sequences have been published elsewhere(Young, Razeghi et al., 2001a; Young, Razeghi et al., 2001b; Peliciari-Garcia, Zanquetta et al., 2011; Bargi-Souza, Romano et al., 2013; Peliciari-Garcia, Previde et al., 2016). Efficiency curves were also performed for all investigated candidate genes; the efficiency/slope values obtained were close to the optimal values required for the 2(-Delta CT) analysis(Dussault & Pouliot, 2006). All quantitative RT-PCR data are normalized to the internal control ribosomal protein L19 (*Rpl19*).

Serum TSH concentration measurements

Serum TSH levels was measured using a specific rat pituitary panel from Milliplex (MAP Rat Pituitary Magnetic Bead Panel - Milliplex, Billerica, MA, USA).

Statistical analysis

Data are presented as means ± SEM. One and two-way ANOVA were applied appropriately to evaluate the influence of time or time and treatment, respectively, on each temporal series, followed by Bonferroni's post-hoc analysis for pair-wise comparisons using GraphPad Prism (GraphPad Software version 6.01, San Diego, CA, USA). A second mathematical and statistical procedure was applied to investigate the presence of a daily 24 h rhythm(Nelson, Tong et al., 1979). The test consisted of adjusting a cosine curve to the real 24 h time series (Cosinor method). The theoretical cosine curve fit was applied to each temporal series using the least-square calculation. It was possible to estimate the fit for each day using F statistics. The null hypothesis tested was that of zero amplitude, that is, no rhythmicity at the assumed frequency (24 h). We considered a periodic fit significant if the *P* value was <0.05. In addition, for each temporal series, the acrophase, mesor and amplitude of the adjusted curve were calculated(Cornelissen, Halberg et al., 1980). When appropriate, Student's *t*-test was also applied. The null hypothesis of no treatment effect was rejected at *P*<0.05.

RESULTS

Experimental protocol validation

The effectiveness of both experimental models was validated through assessment of serum TSH concentration (systemic validation), as well as, *Myh7* mRNA levels in the heart (tissue validation) (Figure 1). Thyroidectomy plus MMI treatment (Tx group) resulted in increased serum TSH concentration and heart *Myh7* expression, as well as decreased *Adrb1* expression (Figs. 1 Ai, ii and iii, respectively), consistent with hypothyroid condition.

Five consecutive days of supraphysiological T3 administration (Hyper group) markedly decreased serum TSH levels and *Myh7* mRNA expression in the heart, as well as increased heart *Adrb1* mRNA levels (Figs. 1 Bi, ii and iii, respectively), consistent with hyperthyroid condition.

Marked effect of hypothyroidism on circadian clock component expression and rhythmicity

Time and hypothyroid condition (treatment) significantly altered the expression of the core clock components *Bmal1*, *Per2*, *Nr1d1* and *Rora* in hypothyroid hearts (Figs. 2 A-Di). An interaction between these variables was observed for *Nr1d1* mRNA content (Fig. 2 Di). Additionally, pair-wise comparisons revealed a reduction of heart core clock component mRNA expression at various time points: *Per2* (ZT 12 and 15, Fig. 2 Bi), *Nr1d1* (ZT 6, Fig. 2 Ci) and *Rora* (ZT 3 and 21, Fig. 2 Di). Cosinor analysis revealed that both mesor and amplitude values for *Bmal1* were reduced in Tx animals (Fig. 2 Aii), while *Per2*, *Nr1d1* and *Rora* (Figs. 2 B-Dii) lost 24-h rhythms in the hypothyroid condition (Table 2).

Loss of diurnal rhythms of metabolic gene expression in hypothyroid hearts

The effects of time and treatment (hypothyroidism) in mRNA expression of genes involved with glucose (*Pdk4*), fatty acid (*Acot1* and *Cd36*), and mitochondrial (*Ucp3*) metabolism were next evaluated (Fig. 3). Both hypothyroidism (treatment) and time significantly altered expression of *Ucp3*, *Pdk4* and *Acot1* (Figs. 3 A-Ci, respectively), while *Cd36* only presented a treatment effect (Fig. 3D). An interaction between time and treatment was observed for *Pdk4* and *Acot1* (Fig. 3 Ci). Pair-wise comparison differences (reductions) were observed at distinct ZTs for *Ucp3* and *Cd36*, ZT 15 (Figs. 3 Ai and D, respectively), *Pdk4* and *Acot1*, ZTs 15, 18 and 21 (Figs. 3 B-Ci, respectively). The expression of *Ucp3*, *Pdk4* and *Acot1* in control hearts exhibited a significant rhythmic oscillation with a periodicity of 24 h (Figs. 3 A-Cii, respectively), while *Cd36* did not present a 24-hr rhythm according to the cosinor analysis (Fig. 3 D). These 24-h rhythms were absent in hearts isolated from hypothyroid animals (Table 2).

Diurnal expression of core clock components in hyperthyroid hearts

Significant time and hyperthyroid treatment main effects were observed for *Bmal1* and *Nr1d1*, while *Rora* and *Per2* mRNA expression presented treatment and time main effect, respectively; interaction between time and treatment main effects was also significant for *Bmal1* and *Rora* (Figs. 4 Ai, Bi, Ci and Di). Pair-wise comparisons showed that T3 excess

reduced *Bmal1* and *Rora* (Figs. 4 Ai and Di, respectively) mRNA contents at ZT 0/24, *Nr1d1* at ZT 9, while no alterations were observed for *Per2* (Fig. 4 Bi and Ci, respectively).

Cosine analysis revealed 24-h rhythms in expression for *Bmal1*, *Per2* and *Nr1d1* in both Control and Hyper groups, while *Rora* exhibited a significant 24-h rhythm only in Control hearts (Figs. 4 A-Dii). Interestingly, the acrophase of *Bmal1* showed a phase delay in hyperthyroid hearts (Fig. 4 Aii); no significant effect of hyperthyroidism on amplitude and mesor were observed for the clock genes investigated (Table 3).

Loss of 24-h rhythmicity of metabolic genes in hyperthyroid hearts

Hyperthyroidism altered the expression of all metabolic genes investigated in the heart (*Ucp3*, *Pdk4*, *Acot1* and *Cd36*; treatment main effect). Time main effect was also observed in the two-way ANOVA for *Acot1* and *Cd36*, with treatment-time interaction additionally observed for *Acot1* mRNA expression (Figs. 5 A-Ci and D). In hyperthyroid hearts, pairwise comparisons revealed that the content of *Ucp3* mRNA at ZTs 3, 6 and 12 (Fig. 5 Ai), as well as, *Acot1* mRNA at ZT 12 were increased (Fig. 5 Ci), while the content of *Cd36* mRNA was significantly reduced at ZT 21 (Fig. 5D), and no time-specific alterations were detected in *Pdk4* mRNA expression, despite the treatment main effect ($P=0.0556$ at ZT 9) (Fig. 5 Bi). Cosinor analysis revealed that *Ucp3*, *Pdk4*, and *Acot1* exhibit significant 24-h rhythms in expression in Control hearts, which are lost following T3 supraphysiological dose treatment (hyperthyroid condition) (Fig. 5 A-Cii) (Table 3).

DISCUSSION

In this study the effects of hypo and hyperthyroidism on the expression of circadian clock and metabolic genes were investigated in the heart. Hypo and hyperthyroid animals presented a predicted pattern of endocrinopathy-induced alterations in TSH secretion and cardiac gene expression of *Myh7* and *Adrb1*. With the exception of *Per2* in hyperthyroid animals, all investigated core clock components and clock-related metabolic genes exhibited significant alterations in response to hypo and hyperthyroidism. Importantly, 24-h oscillations in these genes were dramatically altered during both experimental conditions.

Recently, our group has investigated the acute (<24-h) effects of T3 on the rodent heart circadian clock. In the earlier study, a suggestion of direct T3 action on *Rora* and/or *Per2* was raised due to rapid alterations in these genes (Peliciari-Garcia, Previde et al., 2016). T3 exerts its transcriptional actions through interaction with THR, a ligand-regulated transcription factor, which binds to the thyroid-hormone response element (TRE) present in target genes, mainly as a heterodimer with other nuclear receptors (e.g., retinoid X receptor; RXR). These proteins, along with RORa, belong to the nuclear receptor superfamily. It is noteworthy that these nuclear receptors can undergo reversible covalent modification (in addition to ligand binding), thus impacting activity. For example, RORa is phosphorylated by Ca^{2+} /calmodulin-dependent protein kinases (CaMKs), which increases T3-induced transcription of target genes (Kuno-Murata, Koibuchi et al., 2000).

In the current study, we report that chronic reduction or elevation of circulating T3 levels (hypo and hyperthyroidism, respectively) induced significant alterations in both circadian

clock and metabolic genes in the heart. Of these conditions, hypothyroidism appeared to induce greater alterations, resulting in complete ablation of the circadian rhythmicity of *Per2*, *Nr1d1*, and *Rora*, as well as decreasing the mesor and amplitude of *Bmal1*. Hyperthyroidism also induced alterations in clock component mRNA expression, significantly attenuating the amplitude of *Bmal1*, and abolishing *Rora* rhythm. However, *Per2* and *Nr1d1* rhythms were not significantly influenced by T3 excess, indicating a greater effect of hypothyroidism (vs hyperthyroidism).

During thyroid conditions as hypo and hyperthyroidism changes in behavior, heart metabolism and function are commonly expected. The blood pressure and heart rate are increased in hyperthyroid rats which present ventricular hypertrophy. Food and water intake are further increased in this condition(Wangenstein, Rodriguez-Gomez et al., 2015) On the other hand, its has been described that the natural sex differences of body temperature, body weight gain, and food intake are preserved in the hyperthyroid condition in mice; however the locomotor activity in female mice under the same thyroid condition seems to be increased while muscle strength and motor coordination is reduced in the males. The hypothyroid condition induced higher water intake and serum cholesterol in male mice. Interestingly, the natural sex differences in the heart rate disappeared in both thyroid conditions in mice(Rakov, Engels et al., 2016).

The high energetic demand of the heart for continuous contractile function is met primarily through beta-oxidation of long-chain fatty acids. Changes in fatty acid metabolism have been linked to cardiac pathology in a causal manner. Key fatty acid metabolism proteins include CD36 (an important transporter that facilitates fatty acid uptake by the heart) and cytosolic thioesterase 1 (ACOT1; hydrolyzes cytosolic long-chain fatty acyl-CoAs) (Lopaschuk, Ussher et al., 2010). We have previously suggested that these genes are circadian regulated in the heart. Additional CCGs known to influence glucose and mitochondrial metabolism include pyruvate dehydrogenase kinase 4 (*Pdk4*) and uncoupling protein 3 (*Ucp3*), respectively. Interestingly, these metabolic genes also appear to be regulated by T3(Young, Razeghi et al., 2001b; Shibusawa, Hollenberg et al., 2003).

Here, we confirm that these metabolic genes oscillate over the 24-h day in control hearts (although in the case of *Cd36*, this diurnal pattern did not significantly fit a cosine curve). Importantly, these 24-h rhythms were dramatically altered in both thyroid conditions. We postulate that perturbations in 24-h rhythms of metabolic genes may be due to combined direct (e.g., THR activation and subsequent binding to the TRE) and indirect (e.g., via attenuation of the clock) effect, and that these disrupted rhythms contribute towards perturbations in cardiac metabolism. Whether aberrant diurnal rhythms in the clock and/or metabolic genes play an important role in the pathogenesis of cardiovascular disease warrants future investigation. Furthermore, subclinical thyroid conditions (hypo and hyperthyroidism)(Cerbone, Capalbo et al., 2014; Tadic, Celic et al., 2016; Tadic, Cuspidi et al., 2016) are characterized by alterations in TSH secretion not accompanied by changes in serum T3/T4 concentration. Thus, untreated subclinical thyroid conditions can indeed configure a real risk for cardiovascular function (e.g., lipid metabolic disorders, atherosclerosis, myocardial infarction(Hak, Pols et al., 2000)), as described in the literature(Floriani, Gencer et al., 2017).

It is still worthy mentioning that T3 also interacts with circadian clock in other peripheral tissue. It has been demonstrated that T3 and FSH contributes to the development of circadian clock during granulosa cell maturation (Chu, Misawa et al., 2012). Furthermore, our group is currently investigating the effects of hypo and hyperthyroidism to circadian clock in extra-cardiac tissues. This would provide a better understanding of the systemic effects of the thyroid hormone excess and deprivation to distinct biological systems in the organism.

In summary, mRNA levels of both positive and negative elements of the core clock machinery were altered in the heart during hypo and hyperthyroidism (with the exception of *Per2* for the hyperthyroid condition). Metabolic clock-controlled genes were also affected by the experimental conditions investigated. Interestingly, the clock appears to have a higher tolerance to T3 excess compared to its absence. These findings reveal a new dimension by which T3 modulates gene expression in the heart. We speculate that temporal derangements in transcriptional regulation during hypo and hyperthyroidism may contribute towards cardiovascular disease development.

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Figure 1.

Protocol validation of experimental thyroid conditions. The systemic hypo and hyperthyroid conditions were validated by the serum TSH concentration (Fig. 1 Ai and Bi, $*P < 0.0001$ vs respective controls), while tissues (heart ventricles) validation was evaluated by *Myh7* (Fig. 1 Aii and Bii, $*P = 0.0001$ and $P < 0.0001$ vs respective controls) and *Adrb1* (Fig. 1 Aiii: $P = 0.0461$ and Biii: $P = 0.002$ vs respective controls) mRNA expression. The values are expressed as bar means $\pm$ SD of the 24 h period average expression ($n = 5/\text{ZT}/\text{group}$); Student's unpaired, two-tailed t-test. $n = 5/\text{ZT}/\text{group}$.

Figure 2.

Hypothyroidism and the circadian clock mRNA expression in the rat heart ventricles. The animals were induced hypothyroid by thyroidectomy (Tx, curves and lines in grey color) followed by 2-Mercapto-1-methylimidazole (MMI) 0.03% and CaCl_2 0.05% on drinking water during 20 days treatment and compared to sham-operated animals (Control, curves and lines in black color). A-D) Daily expression of *Bmal1*, *Per2*, *Nr1d1* and *Rora*, respectively, normalized by *Rpl19* mRNA expression, presented as means $\pm$ SEM. (i) Two-way ANOVA, $P < 0.05$ for Tx (treatment) and time main effect, and Tx-time interaction.

Bonferroni's post hoc test, $*P<0.05$ for pair-wise comparisons vs respective ZTs. (ii) Cosinor rhythmic analysis. Control (black line) and Tx (grey dashed line). Differences in mesor (horizontal dashed lines), amplitude and acrophase (capped line) were analyzed using Student *t* test, $*P<0.05$ vs Control mesor, $\#P<0.05$ vs Control amplitude. B-Dii presented no circadian rhythmicity for Tx. $n=5/\text{ZT}/\text{group}$. ZT=Zeitgeber Time.

Figure 3.

Hypothyroidism and metabolic genes mRNA expression in the rat heart ventricles. The animals were induced hypothyroid by thyroidectomy (Tx, curves and lines in grey color) followed by 2-Mercapto-1-methylimidazole (MMI) 0.03% and CaCl_2 0.05% on drinking water during 20 days treatment and compared to sham-operated animals (Control, curves and lines in black color). A-D) Daily expression of *Ucp3*, *Pdk4*, *Acot1* and *Cd36*, respectively, normalized by *Rpl19* mRNA expression, presented as means $\pm$ SEM. (i) Two-way ANOVA, $P < 0.05$ for Tx (treatment) and time main effect, and Tx-time interaction.

Bonferroni's post hoc test, $*P < 0.05$ for pair-wise comparisons vs respective ZTs. (ii) Cosinor rhythmic analysis of Control (black line). *Cd36* presented no circadian rhythmicity for Control and Tx; A-Cii presented no circadian rhythmicity for Tx. $n=5/\text{ZT}/\text{group}$. ZT=Zeitgeber Time.

Figure 4.

Hyperthyroidism and the circadian clock mRNA expression in the rat heart ventricles. The animals were induced hyperthyroid by intraperitoneal injections (i.p.) of saline (Control, curves and lines in black color) or T3 [0.75 $\mu\text{g}/100 \text{ g}$], twice a day / 5 days at ZTs 2 and 14 (Hyper, curves and lines in grey color). A-D) Daily expression of *Bmal1*, *Per2*, *Nr1d1* and *Rora*, respectively, normalized by *Rpl19* mRNA expression, presented as means $\pm$ SEM. (i) Two-way ANOVA, $P < 0.05$ for Hyper (treatment) and time main effect, and Hyper-time interaction. Bonferroni's post hoc test, $*P < 0.05$ for pair-wise comparisons vs respective ZTs.

(ii) Cosinor rhythmic analysis. Control (black line) and Hyper (grey dashed line). Differences in mesor (horizontal dashed lines), amplitude and acrophase (capped line) were analyzed using Student *t* test, & $P < 0.05$ vs Control acrophase. Dii presented no circadian rhythmicity for Hyper. $n=5/\text{ZT}/\text{group}$. ZT=Zeitgeber Time.

Figure 5.

Hyperthyroidism and metabolic genes mRNA expression in the rat heart ventricles. The animals were induced hyperthyroid by intraperitoneal injections (i.p.) of saline (Control, curves and lines in black color) or T3 [0.75 µg/100 g], twice a day / 5 days at ZTs 2 and 14 (Hyper, curves and lines in grey color). A-D) Daily expression of *Ucp3*, *Pdk4*, *Acot1* and *Cd36*, respectively, normalized by *Rpl19* mRNA expression, presented as means ± SEM. (i) Two-way ANOVA, $P < 0.05$ for Hyper (treatment) and time main effect, and Hyper-time interaction. Bonferroni's post hoc test, * $P < 0.05$ for pair-wise comparisons vs respective ZTs.

(ii) Cosinor rhythmic analysis of Control (black line). *Cd36* presented no circadian rhythmicity for Control and Hyper; A-Cii presented no circadian rhythmicity for Hyper. $n=5/\text{ZT}/\text{group}$. ZT=Zeitgeber Time.

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Table 1.

Rat primer sequences used in real-time RT-PCR.

Gene/GenBank#	Primers	Sequences	Amp Length (bp)
<i>Adrb1</i>	Forward	5'-GGGCAACGTGTTGGTGATC-3'	78
NM_012701.1	Reverse	5'-AGGGACATGATGAAGAGGTTGGT-3'	
<i>Acot1</i>	Forward	5'-CACCTGAGGTAAAAGGACCAG-3'	190
NM_031315.2	Reverse	5'-TGGTTTCTCAGGATAGTCACAGG-3'	
<i>Cd36</i>	Forward	5'-ACGACTGCAGGTCAACATAC-3'	113
NM_031561.2	Reverse	5'-CGATGGTCCCAGTCTCATTAG-3'	
<i>Myh7</i>	Forward	5'-AGCGAGGCTCCACCCACAT-3'	206
NM_017240.2	Reverse	5'-CAAGGTGCCCTTGCCTGGGG-3'	

Table 2.

One-way ANOVA and cosinor analysis of Control and hypothyroid (TX) animals. Cosinor parameters are plotted as means $\pm$ SEM; periodicity of 24 h.

One-way ANOVA					Cosinor analysis										
Control			TX		Control						TX				
	P-value	R ²	P-value	R ²	Mesor	Amplitude	Acrophase	P-value	R	Mesor	Amplitude	Acrophase	P-value	R	
<i>Bmal1</i>	0.0001	0.9206	0.0054	0.5191	2.55±0.15	2.92±0.20	1.10±0.17	0.0001	0.9717	1.56±0.20 *	1.84±0.27 *	0.28±0.38	0.002	0.8830	
<i>Per2</i>	0.0125	0.3403	0.1355	0.3396	4.72±0.15	2.26±0.21	14.23±0.22	0.0001	0.9503	x	x	x	x	x	
<i>Nr1d1</i>	0.0001	0.9418	0.0177	0.4631	0.81±0.22	0.98±0.31	9.22±1.14	0.05	0.6290	-	-	-	0.099	0.5373	
<i>Rora</i>	0.0017	0.6196	0.0001	0.6888	1.88±0.12	0.75±0.16	0.21±0.53	0.009	0.7911	-	-	-	0.247	0.3725	
<i>Ucp3</i>	0.0001	0.6499	0.1118	0.3535	1.01±0.12	0.59±0.17	19.21±1.03	0.040	0.6583	x	x	x	x	x	
<i>Pdk4</i>	0.0437	0.2931	0.0003	0.6003	18.16±1.34	9.37±1.98	17.23±0.44	0.009	0.7888	-	-	-	0.123	0.5027	
<i>Acat1</i>	0.0001	0.5784	0.6289	0.1985	0.20±0.03	0.16±0.05	18.32±1.05	0.049	0.6344	x	x	x	x	x	
<i>Cd36</i>	0.5326	0.1393	0.2775	0.2806	x	x	x	x	x	x	x	x	x	x	

One-way ANOVA for each experimental condition (Control and TX). Student's unpaired, two-tailed *t*-test for cosinor analysis

* *P*<0.05 vs respective Control. No variation of the means in One-way ANOVA (x). No 24 h rhythmicity in cosinor analysis (-). Control (Sham operated) and hypothyroid (TX) animals. For optimization of cosinor statistical analysis Mesor and Amplitude values of all groups are multiplied by 100. *n*=5.

One-way ANOVA and cosinor analysis of Control and hyperthyroid (Hyper) animals. Cosinor parameters are plotted as means $\pm$ SEM; periodicity of 24 h.

Table 3.

	One-way ANOVA				Cosinor analysis									
	Control		Hyper		Control					Hyper				
	P-value	R ²	P-value	R ²	Mesor	Amplitude	Acrophase	P-value	R	Mesor	Amplitude	Acrophase	P-value	R
<i>Bmal1</i>	0.0001	0.9419	0.0001	0.7515	1.98±0.37	2.75±0.50	23.21±0.46	0.005	0.8328	1.67±0.17	1.60±0.22	0.33±0.35*	0.001	0.8943
<i>Per2</i>	0.0025	0.6853	0.0001	0.7081	6.22±0.43	3.42±0.59	14.16±0.41	0.003	0.8492	7.08±0.63	5.27±0.86	13.28±0.40	0.003	0.8638
<i>Nr1d1</i>	0.0001	0.9593	0.0001	0.8177	0.86±0.23	1.22±0.34	7.44±1.00	0.032	0.6838	0.60±0.17	0.86±0.24	8.15±1.02	0.032	0.6835
<i>Rora</i>	0.0121	0.3538	0.0134	0.4449	1.81±0.07	0.53±0.10	23.09±0.45	0.004	0.8402	-	-	-	0.128	0.4957
<i>Ucp3</i>	0.0023	0.6882	0.3309	0.2195	0.93±0.10	0.58±0.15	19.47±0.56	0.024	0.7112	x	x	x	x	x
<i>Pdk4</i>	0.0158	0.5980	0.6024	0.1554	23.81±1.74	15.44±2.58	17.33±0.35	0.003	0.8568	x	x	x	x	x
<i>Acot1</i>	0.0008	0.4496	0.0023	0.4709	0.18±0.03	0.13±0.04	18.55±1.05	0.048	0.6362	-	-	-	0.391	0.2685
<i>Cd36</i>	0.0781	0.4949	0.0260	0.4122	x	x	x	x	x	-	-	-	0.519	0.1963

One-way ANOVA for each experimental condition (Control and Hyper). Student's unpaired, two-tailed *t*-test for cosinor analysis

* *P*<0.05 vs respective Control. No variation of the means in One-way ANOVA (x). No 24 h rhythmicity in cosinor analysis (-). Control (Saline) and hyperthyroid (Hyper) animals. For optimization of cosinor statistical analysis Mesor and Amplitude values of all groups are multiplied by 100. *n*=5.