Molecular Neurobiology (2019) 56:7800–7809
https://doi.org/10.1007/s12035-019-1626-y

The Absence of Pineal Melatonin Abolishes the Daily Rhythm of *Tph1* (Tryptophan Hydroxylase 1), *Asmt* (Acetylserotonin *O*-Methyltransferase), and *Aanat* (Aralkylamine *N*-Acetyltransferase) mRNA Expressions in Rat Testes

L. A. Coelho[1] · J. Andrade-Silva[2] · L. C. Motta-Teixeira[2] · F. G. Amaral[3] · R. J. Reiter[4] · J. Cipolla-Neto[2]

Received: 4 January 2019 / Accepted: 25 April 2019 / Published online: 23 May 2019
© Springer Science+Business Media, LLC, part of Springer Nature 2019

Abstract

This study examined the effects of pinealectomy in Wistar rats and melatonin replacement therapy on the daily mRNA expression of melatonin (*Tph1*, *Aanat*, *Asmt*, *Mt1*, *Mt2*, and *Rorα*), and steroidogenic (*Star*, *17βhsd3*, and *Lhr*) related genes as well as clock genes (*Rev-erbα*, *Bmal1*, *Per1*, *Per2*, *Cry1*, and *Cry2*) in testes. The testes of control animals express the *Tph1*, *Aanat*, and *Asmt* and *Per2* genes with 24-h rhythms in mRNA, reaching the maximal values during the dark phase. Pinealectomy abolished and melatonin treatment restored the 24-h rhythmicity. Daytime differences in mRNA expression were significant for *Star*, *Lhr*, *Mt1*, *Mt2*, *Rorα*, *Rev-erbα*, *Bmal1*, *Cry1*, and *Cry2* genes in testes of control rats. Conversely, *17βhsd3* and *Per1* mRNA expression did not show a daytime difference in testes of control animals. Pinealectomy abolished the peak time of *Mt1* and *Mt2* mRNA expression, phase shifted the peak time of *Star*, *Rorα*, *Rev-erbα*, *Bmal1*, and *Cry2* mRNA expression, downregulated the 24-h *Lhr* mRNA expression, and inverted the peak time of *Per1*, *Per2*, and *Cry1* mRNA expression to the light phase. The melatonin replacement therapy completely restored the control levels of *Lhr*, *Rev-erbα*, and *Per1* mRNA expression patterns, partially restored the daily control of *Star*, *Mt2*, *Rorα*, *Bmal1*, *Cry1*, and *Cry2* mRNA expression but did not re-establish the daily control of *Mt1* mRNA expression. This suggests that the daily mRNA expression of these genes is probably driven by pineal melatonin and melatonin treatment restores (partially or completely) the daily control of gene expression patterns.

Keywords Clock genes · Melatonin receptors · Steroidogenic enzymes · Testes

Introduction

The pineal melatonin rhythm is usually associated with the modulation of circadian rhythms [1, 2]. Pineal melatonin synthesis involves tryptophan hydroxylation of by tryptophan hydroxylase 1 (TPH1) and its decarboxylation by dopa decarboxylase (DDC, also known as aromatic L-amino acid descarboxylase) leads to the formation of serotonin [3]. The conversion of serotonin to melatonin involves the acetylation of serotonin into *N*-acetylserotonin (NAS) by arylalkylamine *N*-acetyltransferase (AANAT) and the conversion of NAS to melatonin by the acetylserotonin *O*-methyltransferase (ASMT). Both AANAT and ASMT are considered rate-limiting enzymes for melatonin synthesis [4].

Melatonin is synthesized and released by pineal gland at night and its secretion is limited to the dark phase of the light-dark cycle. The duration of melatonin secretion transforms the circadian information into a chemical message that is used by the organism [5]. The circadian pattern of pineal melatonin secretion is regulated by clock genes localized in the suprachiasmatic nuclei (SCN) of the hypothalamus [6]. Melatonin has been also implicated to the regulation of the expression of clock genes in most tissues of the body [7–10]. There is also evidence of non-pineal sources of melatonin [11] including

✉ L. A. Coelho
liac@usp.br

[1] Department of Veterinary Medicine, Faculty of Animal Science and Food Engineering (FZEA), University of São Paulo (USP), Av. Duque de Caxias Norte, 225, Pirassununga, SP 13635-900, Brazil

[2] Department of Physiology and Biophysics, Institute of Biomedical Sciences (ICB), University of São Paulo (USP), São Paulo SP, Brazil

[3] Department of Physiology, Federal University of São Paulo (UNIFESP), São Paulo SP, Brazil

[4] Department of Cellular and Structural Biology, University of Texas Health Science Center, San Antonio, TX, USA

the reproductive organs where local *Aanat* and *Asmt* mRNA and protein expressions [12–15] and AANAT and ASMT activities [16, 17] in the females [12–14, 17] and males [15, 16] have been detected.

Melatonin also plays an important role in the regulation of testicular function including testosterone secretion [18]. Melatonin influences the mRNA expression of protein and enzymes that are essential for testosterone synthesis including steroidogenic acute regulatory protein (*Star*) and *17βHsd3* [19, 20]. In addition, it has been also postulated that melatonin acts as a local antioxidant neutralizing free radical-mediated damage [21].

Despite many studies indicating that melatonin has an impact on testicular function, the mechanism of action remains unknown. It was reported that the effects of melatonin on testicular function may involve binding sites for melatonin [22]. Membrane melatonin receptors, such as MT1 and MT2, have been identified in testes of adult rats and during several developmental ages [23]. Nuclear binding sites for melatonin including RORα [24] also have been detected in testes [25]. Additionally, it was demonstrated that the mRNA expression of clock genes, melatonin-forming enzyme, and melatonin receptor genes in peripheral tissues are under the control of the circulating melatonin [13, 26].

In the present study, we investigated, in rat testes, the daily mRNA expression of melatonin synthesizing enzyme genes (*Tph1*, *Aanat*, and *Asmt*), membrane (*Mt1* and *Mt2*), and the putative nuclear (*Rorα*) melatonin receptor genes, as well as the clock genes (*Bmal1*, *Rev-erbα*, *Cry1*, *Cry2*, *Per1*, and *Per2*) and testosterone synthesis-related genes (*Lhr*, *Star*, and *17βHsd3*). In addition, we examined the effect of pinealectomy and melatonin replacement therapy, on the diurnal mRNA expression of these genes in the testes.

Materials and Methods

Animals

Male adult Wistar rats obtained from the Institute of Biomedical Sciences, University of São Paulo, Brazil, were kept on a 12L:12D light-dark cycle, in a temperature-controlled room (23 °C ± 2 °C), with food and water available ad libitum. The Animal Care Committee of Ethics in Animal Experimentation of the Institute of Biomedical Sciences, University of São Paulo, approved all the procedures involving animals.

Pinealectomy

The animals were anesthetized with intraperitoneal injection of ketamine and xilazim (0.15 mL/100 g body weight) and submitted to the surgery according to Hoffman and Reiter's method [27]. The anesthetized animal was fixed in a stereotaxic apparatus and the scalp was opened in a sagittal manner. To expose the lambda suture, the skin and muscles were pushed aside. By means of a circular drill, a disc-shaped bone was loosened over the lambda and the loosen bone was delicately removed. Thereafter, the superficial pineal gland was removed with a fine forceps. After a brief hemostasis, the skull was closed by returning the disc-shaped bone and the scalp was sutured with cotton suture.

Melatonin Treatment

Melatonin (Sigma-Aldrich, Saint Louis, MO, USA) solution was prepared according to the individual daily water intake and the body weight (1 mg/kg body weight). Melatonin solution was daily added to drinking water and given to the animals only during the 12-h of the dark phase of the light-dark illumination cycle. At the beginning of the 12-h light phase, the bottles were replaced by others with tap water free of melatonin. The melatonin treatment lasted 4 weeks.

mRNA Extraction and cDNA Synthesis

Total RNA was extracted from testes using guanidine isothiocyanate-based reagent (TRIzol®, Invitrogen, Carlsbad, USA) according to the manufacturer's specifications. The cDNA was synthetized from 1 μg of total RNA in a total reaction volume of 20 μL by using reverse transcriptase (200 U Superscript III, Invitrogen, Carlsbad, USA), DTT (10 nM), dNTP (10 nM each), RNase inhibitor (40 U), and random primers (150 ng).

Real-Time Quantitative PCR

Quantitative reverse transcriptase polymerase chain reaction (qRT-PCR) was performed (7500 Real-Time PCR System; Applied Biosystems, Inc., [ABI] Foster City, CA) with 12 μL containing 1 μL cDNA, SYBR green (Power SYBR green, ABI), and 200 nM specific primers (Table 1). For *Tph1*, *Aanat*, *Asmt*, *Mt2*, *Bmal1*, *Rev-erbα*, *Cry1*, *Cry2*, *Per1*, and *Per2* mRNA expression, an absolute quantitative analyses were performed and a set of 10-fold serial dilutions of internal standard (10^2–10^6 copies/1 μL) was used to generate an absolute standard curve, and all qRT-PCR assays linear within this concentration range with correlation coefficients (r^2) > 0.999. The relative expression of *Mt1*, *Rorα*, *Star*, *Lhr*, and *17βHsd3* mRNAs was calculated, using the relative analyses by the $2^{-\Delta\Delta CT}$ method [28] and reported as arbitrary units. All measures were performed in duplicate for each sample and *Rpl37a* was indicated as most suitable reference gene by the software GeNorm [29] since the amount of *Rpl37a* mRNA did not vary according to the time.

Experimental Design

Animals were divided in three groups: sham-operated (CONTROL), pinealectomized (PINX), and pinealectomized melatonin-treated (PINX-MEL). Animals (three to five animals per time point per group) were euthanized by decapitation every 3 h throughout a 24-h light-dark cycle. The testicular samples were collected at following zeitgeber times (ZTs): ZT3 (3 h after light on), ZT6, ZT9, ZT12, ZT15 (3 h after light off), ZT18, ZT21, and ZT24.

Statistical Analysis

Quantitative RT-PCR results were plotted as mean ± SEM, calculated from at least three replications. The differences among ZTs within each experimental group (CONTROL, PINX, and PINX-MEL) were analyzed using one-way ANOVA and comparisons between groups were done using two-way ANOVA followed by Bonferroni's post-test. When appropriate, Student's *t* test was performed. These analyses were carried out using GraphPad Prism (GraphPad Software version 7.00; San Diego, CA, USA). To study the presence of 24-h rhythmicity, a cosinor analysis was applied [30]. To determine the peak time of mRNA expression of those genes that daily profile of expression was not adjusted to a cosinor curve, an ordinary one-way ANOVA analysis was performed. It was considered the peak time of expression the time (ZT) in whose high level of expression was significantly different to the all the other times (ZTs).

Results

As seen in Fig. 1 and in Tables 2 and 3, in the peripheral testicular tissue, three essential melatonin-synthetizing enzyme (*Tph1*, *Aanat*, and *Asmt*) gene expression in CONTROL rats show a clear 24-h rhythm variation peaking during the night (Fig. 1 and Table 3). Most interestingly, the absence of pineal melatonin in PINX animals either abolished the daily rhythm of mRNA expression of *Tph1* (Fig. 1a) and

Table 1 List of qRT-PCR primers used in the present research

Target and reference genes	Accession number[a]	Primer sequence 5′–3′	Base pairs (bp)
Mt1	NM_053676.1	F: 5′ – CGTGGTGGACATCCTGGG − 3′ R: 5′ – CGAGGTCTGCCACAGCTAAACT − 3′	109
Mt2	NM_001100641.1	F: 5′ – TCCTCTCGGTGCTCAGGAAC – 3′ R: 5′ – AGGTCAGCCAAGGCCAGATT–3′	75
Rorα	NM_001106834.1	F: 5′ – CAGGCAGAGCTATGCGAGC – 3′ R: 5′ – TCCACAGATCTTGCATGGAATAA − 3′	93
Tph1	NM_001100634	F: 5′ – CTCTTGGAGCTTCAGAGGAGAC – 3′ R: 5′ – GACTCTCAGCTGCCCATCTTG − 3′	98
Asmt (Hiomt)	NM_144759.2	F: 5′ – TGCCCGCACCCACTTCCTGTC – 3′ R: 5′ – GACCCGGGCAAGAATGAAGAG − 3′	112
Aanat	NM_012818	F: 5′ – AAAGTACACTCAGGCACCAATGT − 3′ R: 5′ – GGGAACATAGCTGCTTTATTAGTGTCAG − 3′	110
Bmal1	NM_024362.2	F: 5′ – CCGATGACGAACTGAAACACC – 3′ R: 5′ – TCTTCCCTCGGTCACATCCT − 3′	77
Rev-erbα	NM_145775.1	F: 5′ – AGGTGACCCTGCTTAAGGCTG − 3′ R: 5′ – ACTGTCTGGTCCTTCACGTTGA − 3′	81
Cry1	NM_198750.2	F: 5′ – TTCGCCGGCTCTTCCAA − 3′ R: 5′ – ATTGGCATCAAGGTCCTCAAGA − 3′	74
Cry2	NM_133405.1	F: 5′ – TCAGCGTGAATGCAGGCA − 3′ R: 5′ – AGGGCAGTAGCAGTGGAAGAAC – 3′	76
Per1	NM_001034125.1	F: 5′ – CTGCCTCAGGCCCTCGA − 3′ R: 5′ – GTCCGAGTGGCCAGGATCTT − 3′	71
Per2	NM_031678.1	F: 5′ – GCAGCCTTTCGATTATTCTCCC – 3′ R: 5′ – GGACCAGCTAGTGTCCAGTGTG − 3′	75
Star	NM_031558.3	F: 5′ – ATTGACCTCAAGGGGTGGCT – 3′ R: 5′ – GCTGGCGAACTCTATCTGGG – 3′	75
17βhsd3	NM_054007.1	F: 5′– CATTATCCAGGTGCTGACCCC – 3′ R: 5′ – AAACTCATCGGCGGTCTTGG – 3′	91
Lhr	NM_012978.1	F: 5′ – TTCCCAGGAGCAAGTAAGCC – 3′ R: 5′– TAACGCTCTCGGTGGTATGG − 3′	84
Rpl37a	NM_001108801	F: 5′ – TTGAAATCAGCCAGCACGC – 3′ R: 5′ – TGCCAACGGCTCGTCTCT − 3′	74

F forward, *R* reverse

[a] Accession number is provided by the National Center for Biotechnology Information, Bethesda, MD

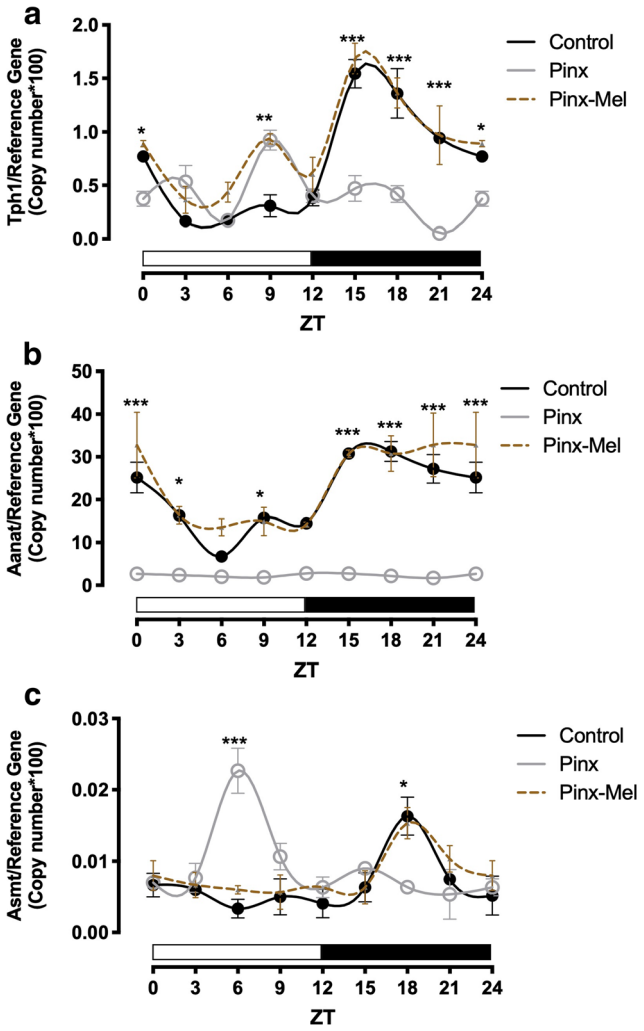

Fig. 1 Daily mRNA expression of *Tph1* (**a**), *Aanat* (**b**), and *Asmt* (**c**) genes in testes of sham-pinealectomized (CONTROL), pinealectomized (PINX), and pinealectomized melatonin-treated rats (PINX-MEL). A two-way ANOVA with Bonferroni's multiple comparisons post-test was used to determine significant differences between values (plotted as mean ± SEM). Asterisks represent CONTROL vs. PINX differences (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$). The black bar represents the dark phase of the light-dark cycle. ZT, zeitgeber time; ZT0 = ZT24. Reference gene: *Rpl37a*

Aanat (Fig. 1b) genes or phase shifted the daily peak of *Asmt* to daytime (Fig. 1c). Melatonin replacement therapy in PINX-MEL animals re-established the 24-h rhythm and the daily peak nocturnal phase of mRNA levels of the three enzymes (Table 3) but not blunted the diurnal peak of *Tph1* expression provoked by pinealectomy (Table 2).

The mRNA expression of the three testicular steroidogenesis-related genes showed a clear daily variation peaking during the night in the CONTROL rats for *Star* (Fig. 2a) and *Lhr* (Fig. 2c) genes. In the case of *Lhr* gene expression, the absence of circulating melatonin due pinealectomy abolished the daily variation and its replacement (PINX-MEL) re-established the daily profile of mRNA expression (Table 2). In the case of the *Star* gene expression, the absence of pineal melatonin phase shifted the daily peak to earlier times during the night (Figs. 2a and 5) while melatonin replacement prevented this early time peak but did not re-establish the peak at latter times (Table 2). The daily mRNA expression of *17βhsd3* gene (Fig. 2b) in testes of CONTROL animals did not show any statistical variation during the 24-h and this daily distribution was not altered by pinealectomy without or with a melatonin treatment (Table 2).

Figure 3 and Table 4 document a significant daily distribution of testicular membrane and putative nuclear melatonin receptors gene expression. The *Mt1* (Fig. 3a) and *Rorα* (Fig. 3c) mRNA expression peaks occurred in the first half of the light phase and *Mt2* (Fig. 3b) mRNA expression peaks at the middle of the dark phase of the light-dark cycle. In the case of *Mt1* and *Mt2*-melatonin receptors, the absence of pineal melatonin in testes of PINX rats abolished these daily variations and therapeutic melatonin replacement (as seen in PINX-MEL animals) either did not re-establish the daily variation, as is the case for the *Mt1*, or only partially recovered its amplitude as seen for *Mt2*-melatonin receptor (Table 4). In the case of testicular *Rorα* gene expression (Figs. 3c and 5), the absence of pineal melatonin provoked a phase displacement of its daily peak from dawn to dusk of the light phase. Melatonin replacement was only able to partially prevent the phase-displacement.

In the case of testicular clock gene expressions (Fig. 4 and Table 5), it is noteworthy that the particular daily distribution of each of them (*Bmal1*, *Per1*, *Per2*, *Cry1*, *Cry2*, and *Rev-erbα*) changed considerably in the absence of circulating melatonin (PINX rats) and was almost completely restored to the CONTROL pattern with melatonin replacement therapy (PINX-MEL rats). The replacement of plasma melatonin in pinealectomized animals (Table 5) completely restored the daily mRNA expression profiles of *Rev-erb* and *Per1* genes and weakly restored the mRNA expression of *Bmal1* (ZT3), *Cry1* (ZT3 and ZT6), and *Cry2* (ZT3 and ZT24) genes. However, the peak time of mRNA expression of *Bmal1* gene in testes of CONTROL animals displaced by pinealectomy was restored with melatonin treatment (Fig. 5). A significant circadian rhythm of mRNA expression in testes of CONTROL rats was observed only for *Per2* gene (Table 5). Pinealectomy abolished the 24-h rhythmicity of mRNA expression of this gene and melatonin replacement restored the circadian rhythm to that as observed in testes of CONTROL animals.

Discussion

The present study documents the daily mRNA expression of the melatonin-forming enzymes (*Tph1*, *Aanat*, and *Asmt*), melatonin membrane (*Mt1* and *Mt2*) and putative nuclear

Table 2 Results of two-way ANOVA with Bonferroni's multiple comparisons post-test of daily mRNA expression of melatonin-forming enzymes (*Tph1*, *Aanat*, and *Asmt*) and steroidogenesis-related genes (*Star*, *17βhsd3*, and *Lhr*) in testes of sham-pinealectomized (C), pinealectomized (P), and pinealectomized melatonin-treated (M) rats

Gene/ ZT	C vs. P	C vs. M	P vs. M	C vs. P	C vs. M	P vs. M	C vs. P	C vs. M	P vs. M
	Tph1			*Aanat*			*Asmt*		
LP									
ZT3	NS	NS	NS	*	NS	*	NS	NS	NS
ZT6	NS	NS	NS	NS	NS	NS	***	NS	***
ZT9	**	**	NS	*	NS	*	NS	NS	NS
ZT12	NS	NS	NS	NS	NS	NS	NS	NS	NS
DP									
ZT15	***	NS	***	***	NS	***	NS	NS	NS
ZT18	***	NS	***	***	NS	***	**	NS	*
ZT21	***	NS	***	***	NS	***	NS	NS	NS
ZT24	*	NS	*	***	NS	***	NS	NS	NS
	Star			*17βhsd3*			*Lhr*		
LP									
ZT3	NS	NS	NS	NS	NS	NS	*	NS	*
ZT6	NS	NS	NS	NS	NS	NS	NS	NS	NS
ZT9	NS	NS	NS	NS	NS	NS	NS	NS	NS
ZT12	NS	NS	NS	NS	NS	NS	NS	NS	NS
DP									
ZT15	NS	NS	NS	NS	NS	NS	NS	NS	NS
ZT18	***	NS	***	NS	NS	NS	NS	NS	NS
ZT21	***	NS	NS	NS	NS	NS	***	NS	***
ZT24	NS	NS	NS	NS	NS	NS	NS	NS	NS

Asterisks represent significant differences (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$)

NS non-significant, *LP* light phase, *DP* dark phase

Table 3 Cosinor analysis of mRNA expression of melatonin-forming enzymes (*Tph*, *Aanat*, and *Asmt*) and *Per2* genes in testes of sham-pinealectomized (CONTROL), pinealectomized (PINX), and pinealectomized melatonin-treated (PINX-MEL) rats

Genes	Acrophase[a]	Amplitude	Mesor	Cosinor 24-h analysis (*P* values)
Tph				
CONTROL	17.8 ± 0.329*	0.657 ± 0.035	0.703 ± 0.009*	$P = 0.010$
PINX	–	–	–	$P = 0.462$
PINX-MEL	16.8 ± 0.170	0.563 ± 0.066	0.910 ± 0.045	$P = 0.049$
Aanat[b]				
CONTROL	18.6 ± 0.550	2.077 ± 0.039	20.98 ± 1.262	$P = 0.007$
PINX-MEL	19.7 ± 0.663	3.280 ± 0.168	19.51 ± 0.770	$P = 0.006$
Asmt				
CONTROL	19.3 ± 0.258	0.603 ± 0.092	0.627 ± 0.061	$P = 0.027$
PINX	–	–	–	$P = 0.246$
PINX-MEL	18.5 ± 0.867	0.396 ± 0.104	0.750 ± 0.063	$P = 0.031$
Per2				
CONTROL	19.9 ± 0.051**	16.43 ± 0.703*	18.84 ± 0.687	$P = 0.012$
PINX	–	–	–	$P = 0.970$
PINX-MEL	19.3 ± 0.088	9.46 ± 0.792	19.53 ± 0.877	$P = 0.073$

Asterisks represent significant differences (*$P < 0.05$, **$P < 0.01$) by Student's *t* test

[a] Acrophase is expressed as zeitgeber time (ZT)

[b] There were no time differences ($P > 0.05$) in *Aanat* mRNA expression in testes of PINX rats then no cosinor 24-h analysis was performed

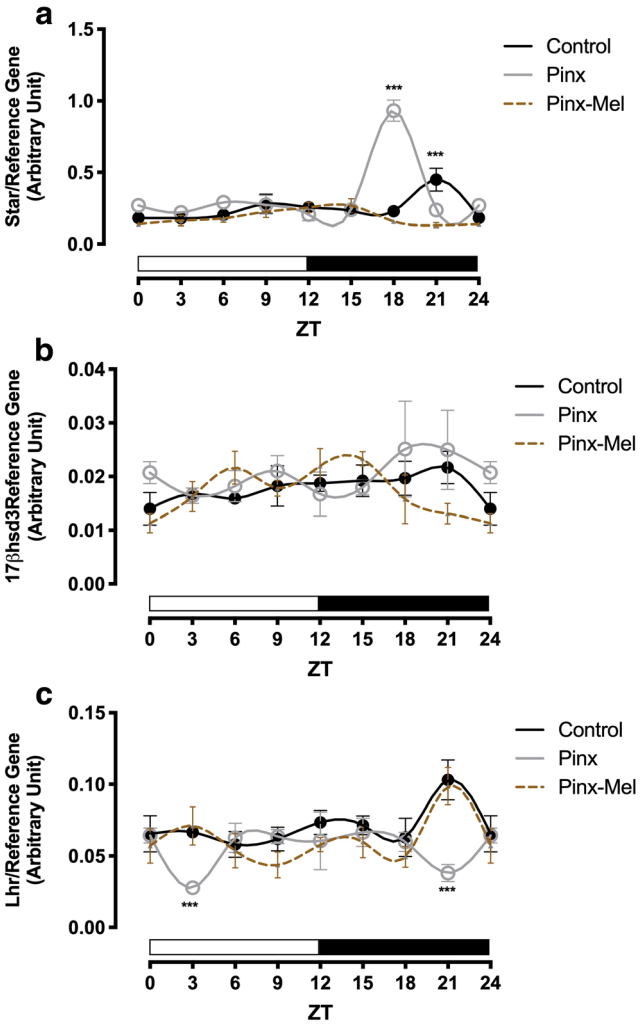

Fig. 2 Daily mRNA expression of *Star* (**a**), *17βhsd3* (**b**), and *Lhr* (**c**) genes in testes of sham-pinealectomized (CONTROL), pinealectomized (PINX), and pinealectomized melatonin-treated rats (PINX-MEL). A two-way ANOVA with Bonferroni's multiple comparisons post-test was used to determine significant differences between values (plotted as mean ± SEM). Asterisks represent CONTROL vs. PINX differences (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$). The black bar represents the dark phase of the light-dark cycle. ZT, zeitgeber time; ZT0 = ZT24. Reference gene: *Rpl37a*

(*Rorα*) receptor genes, clock genes (*Rev-erbα*, *Bmal1*, *Cry1*, *Cry2*, *Per1*, and *Per2*) and steroidogenic-related (*Star*, *17βHsd3*, and *Lhr*) genes in rat testes. We also demonstrated the effect of pinealectomy and melatonin replacement therapy on daily mRNA expression of these genes.

The testes of CONTROL animals express the melatonin-forming enzyme (*Tph1*, *Aanat*, and *Asmt*) genes with 24-h rhythm in mRNA levels similar for each gene, reaching the maximal values during the dark phase (ZT17 to ZT19). Pinealectomy abolished these rhythmic patterns by blunting the nocturnal mRNA profiles and melatonin treatment restored the 24-h rhythmicity model of *Tph1*, *Aanat*, and *Asmt* mRNA expression as observed in testes of CONTROL animals. The presence of transcripts of melatonin-forming enzyme genes in testes indicates possible local melatonin synthesis, which remarkably, is under the daily control of pineal circulating melatonin. The evidence that testes synthetize melatonin was well documented [31] and is supported by the presence of high enzymatic activity of ASMT and AANAT in interstitial cells [16] and high protein expression of ASMT and AANAT in Leydig cells, spermatocytes, and spermatids [15]. Moreover, the mitochondria of all cells, including those of the testes, are predicted to produce melatonin [21]. These data suggest that local melatonin secretion could participate in different physiological processes in testes, similar to those observed in the ovary. It was observed that melatonin exists in the seminal plasma [32] produced by the testes [15] and has the function to protect the spermatozoa from oxidative damage, which influences the male fertility [33]. Local melatonin

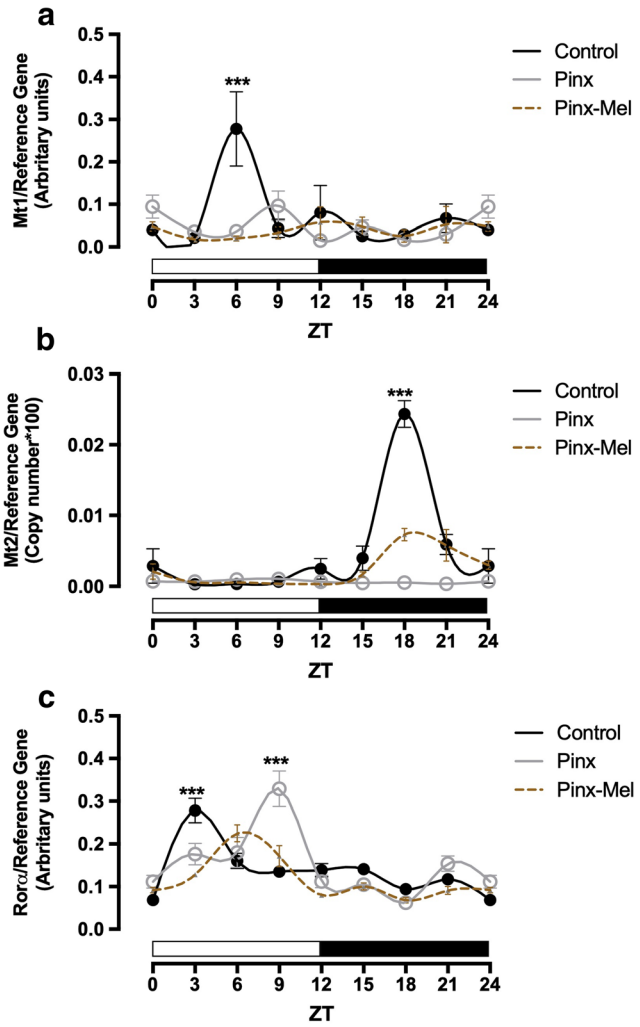

Fig. 3 Daily mRNA expression of *Mt1* (**a**), *Mt2* (**b**), and *Rora* (**c**) genes in testes of sham-pinealectomized (CONTROL), pinealectomized (PINX), and pinealectomized melatonin-treated (PINX-MEL) rats. A two-way ANOVA with Bonferroni's multiple comparisons post-test was used to determine significant differences between values (plotted as mean ± SEM). Asterisks represent CONTROL vs. PINX differences (***$P < 0.001$). The black bar represents the dark phase of the light-dark cycle. ZT, zeitgeber time; ZT0 = ZT24. Reference gene: *Rpl37a*

Table 4 Results of two-way ANOVA with Bonferroni's multiple comparisons post-test of daily mRNA expression of melatonin receptors (*Mt1*, *Mt2*, and *Rorα*) in testes of sham-pinealectomized (C), pinealectomized (P), and pinealectomized melatonin-treated (M) rats

Gene/ZT	C vs. P	C vs. M	P vs. M	C vs. P	C vs. M	P vs. M	C vs. P	C vs. M	P vs. M
	Mt1			*Mt2*			*Rorα*		
LP									
ZT3	NS	NS	NS	NS	NS	NS	NS	***	NS
ZT6	***	***	NS	NS	NS	NS	NS	NS	NS
ZT9	NS	NS	NS	NS	NS	NS	***	NS	***
ZT12	NS	NS	NS	NS	NS	NS	NS	NS	NS
DP									
ZT15	NS	NS	NS	NS	NS	NS	NS	NS	NS
ZT18	NS	NS	NS	***	***	***	NS	NS	NS
ZT21	NS	NS	NS	**	NS	**	NS	NS	NS
ZT24	NS	NS	NS	NS	NS	NS	NS	NS	NS

Asterisks represent significant differences (**$P < 0.01$, ***$P < 0.001$)

NS non-significant, *LP* light phase, *DP* dark phase

secretion could act as an intracellular mediator or a paracrine signal in testicular steroidogenic processes by downregulating the steroidogenic acute regulatory (StAR) protein [34], which is the rate-limiting step in steroidogenesis [35].

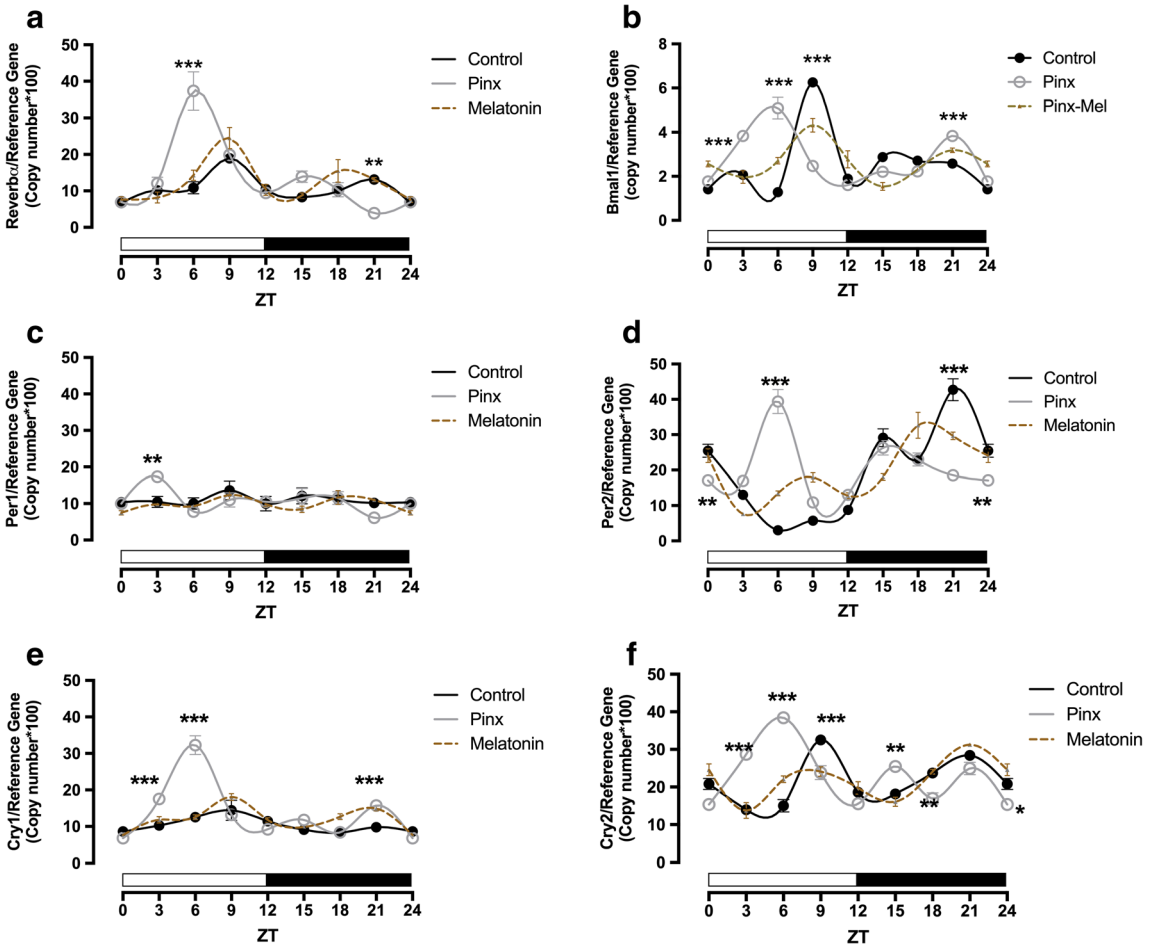

Fig. 4 Daily mRNA expression of *Rev-erbα* (**a**), *Bmal1* (**b**), *Per1* (**c**), *Per2* (**d**), *Cryl* (**e**), and *Cry2* (**f**) genes in testes of sham-pinealectomized (CONTROL), pinealectomized (PINX), and pinealectomized melatonin-treated (PINX-MEL) rats. A two-way ANOVA with Bonferroni's multiple comparisons post-test was used to determine significant differences between values (plotted as mean ± SEM). Asterisks represent CONTROL vs. PINX differences (**$P < 0.01$, ***$P < 0.001$). The black bar represents the dark phase of the light-dark cycle. ZT, zeitgeber time; ZT0 = ZT24. Reference gene: *Rpl37a*

Table 5 Results of two-way ANOVA with Bonferroni's multiple comparisons post-test of daily mRNA expression of clock genes (*Rev-erbα*, *Bmal1*, *Per1*, *Per2*, *Cry1*, and *Cry2*) in testes of sham-pinealectomized (C), pinealectomized (P), and pinealectomized melatonin-treated (M) rats

Gene/ ZT	C vs. P	C vs. M	P vs. M	C vs. P	C vs. M	P vs. M	C vs. P	C vs. M	P vs. M
	Rev-erbα			*Bmal1*			*Per1*		
LP									
ZT3	NS	NS	NS	***	NS	***	**	NS	**
ZT6	***	NS	***	***	***	***	NS	NS	NS
ZT9	NS	NS	NS	***	***	***	NS	NS	NS
ZT12	NS	NS	NS	NS	*	***	NS	NS	NS
DP									
ZT15	NS	NS	NS	NS	***	NS	NS	NS	NS
ZT18	NS	NS	NS	NS	NS	NS	NS	NS	NS
ZT21	***	NS	***	***	NS	NS	NS	NS	NS
ZT24	NS	NS	NS	NS	***	*	NS	NS	NS
	Per2			*Cry1*			*Cry2*		
LP									
ZT3	NS	NS	**	***	NS	***	***	NS	***
ZT6	***	***	***	***	NS	***	***	**	***
ZT9	NS	***	*	NS	NS	**	***	***	***
ZT12	NS	NS	NS	NS	NS	NS	NS	NS	NS
DP									
ZT15	NS	***	***	NS	NS	NS	**	NS	***
ZT18	NS	***	***	NS	*	*	**	NS	**
ZT21	***	***	***	***	**	NS	NS	NS	*
ZT24	**	NS	*	NS	NS	NS	*	NS	***

Asterisks represent significant differences (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$)

NS non-significant, *LP* light phase, *DP* dark phase

Our findings demonstrated that pinealectomy altered the daily *Star* and *Lhr* mRNA expressions. The absence of circulating melatonin provokes a displacement of the daily peak of *Star* mRNA expression to early times during the dark phase and melatonin replacement therapy restored the daily pattern distribution as seen in the CONTROL animals. In contrast, the daily pattern of testicular *Lhr* mRNA expression, altered by pinealectomy was completely restored by melatonin replacement therapy. Daily variation of *Star* mRNA expression was previously observed in rat testis and its profile was similar to the daily testicular testosterone levels [36]. It was also observed that there is a seasonal variation of LH receptor site bindings in rat testis, and after pineal removal, the seasonal changes are lost [37]. In the testis, testosterone production is

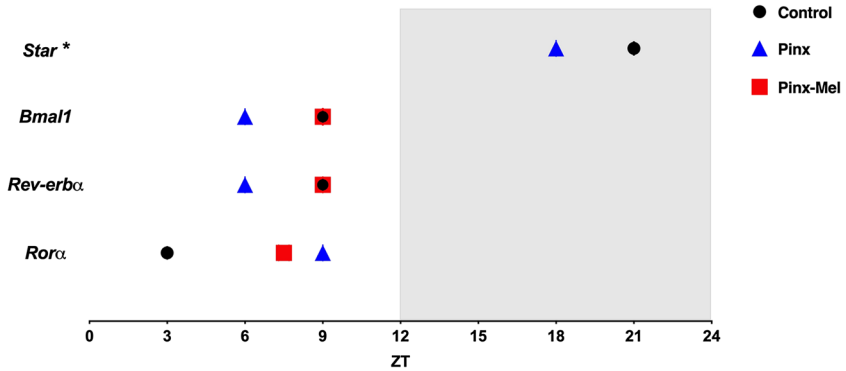

Fig. 5 Peak time mRNA expression of *Rorα*, *Bmal1*, *Rev-erbα*, and *Star* genes in testes of sham-pinealectomized (CONTROL), pinealectomized (PINX), and pinealectomized melatonin-treated (PINX-MEL) rats. The peak time of expression was calculated by a one-way ANOVA analysis considering the ZT in whose high level of expression was significantly different to the all the other ZTs within each group (CONTROL, PINX, and PINX-MEL). There were no time differences (*$P > 0.05$) in *Star* mRNA expression in testes of PINX-MEL rats. The gray square represents the dark phase of the light-dark cycle

predominantly governed by initiation of cAMP signaling pathway due to binding of LH to its receptor coupled to G protein regulating adenylyl cyclase by increasing production of cAMP and subsequent activation of StAR protein which aids the transport of free cholesterol to the inner mitochondrial membrane where the first step of steroidogenesis occurs [38]. It has been demonstrated that melatonin reduce the stimulation of StAR protein expression by LH or cAMP without altering testosterone production, a physiological process mediated by membrane melatonin receptors [39].

The daily *Mt1*, *Mt2*, and *Rorα* mRNA expression profiles in rat CONTROL testes showed a marked 24-h variation with different peak time of expression for each gene. For *Mt1* and *Rorα* genes, the peak time of mRNA expression occurred during the light phase while the peak time of *Mt2* mRNA expression was in the dark phase. Our findings also demonstrated that pinealectomy altered testicular daily RNA expression profiles of *Mt1* and *Mt2* genes suggesting that these daily gene expression profiles are, in some way, controlled by circulating melatonin. The absence of pineal melatonin abolished the peak time of *Mt1* and *Mt2* mRNA expression and phase-displaced the peak time of *Rorα* mRNA expression. Melatonin replacement did not restore (*Mt1*) or only partially restored (*Mt2* and *Rorα*) the gene expression to the levels observed in testes of control animals. Previously, it had been demonstrated that pineal melatonin influences the expression of melatonin receptor genes in rat liver [26] and cumulus-oocyte complexes [13] and the absence or presence of melatonin in the blood either up- or downregulated the melatonin receptors in a different manner. In fact, the presence of mRNA expression of melatonin receptors has been detected in several testicular cells [23, 39] including in the spermatozoa of several seasonal and non-seasonal breeders [40]. These receptors are responsible for mediating some melatonin actions in testes such as androgen production [19, 39] and protection of spermatozoa against damage-induced apoptosis [41] or they act as a modulator of circadian clock mechanism [42, 43].

Our study revealed that the mRNA expression of clock genes in the testis show a characteristic daily curve for each one of the studied genes. It should be noted that a total testis preparations were used and the obtained qPCR daily change is a reflection of the all daily changes in the different testicular cell types. In any case, however, it is possible to see that the absence of pineal melatonin altered the daily distribution of all the studied clock genes and that melatonin replacement therapy restored the daily pattern to the level of the CONTROL animals. It has been demonstrated that male clock *Bmal1* knockout mice present a reduced plasma testosterone and sperm count [44]. In fact, the male clock *Bmal1* knockout mice are infertile mainly due to the reduction of the expression of steroidogenic genes in testes including the expression of *Star* gene [45].

One physiological consequences of the absence of circulating pineal melatonin in rat testes could be gonadal hypertrophy [46] and altered testosterone secretion, which may be seasonally time dependent [37] since exogenous melatonin treatment had a positive impact of blood testosterone levels [47].

In conclusion, the melatonin cycle of secretion from the pineal gland has a major role in regulating the daily pattern of mRNA expression of most genes studied suggesting that the absence of melatonin in the circulation could alter the normal molecular physiology of the rat testes.

Funding This work was financially supported by the Fundação de Amparo à Pesquisa do Estado de São Paulo, FAPESP (2014/17830-9), FAPESP (2014/50457-0), São Paulo, Brazil.

Compliance with Ethical Standards

Conflict of Interest The authors declare that they have no conflict of interest.

References

1. Reiter RJ, Fraschini F (1969) Endocrine aspects of the mammalian pineal gland: a review. Neoroendocrinology 5:219–255
2. Cipolla-Neto J, Amaral FG (2018) Melatonin as a hormone: new physiological and clinical insights. Endocr Rev 39:990–1028
3. Rath MF, Coon ST, Amaral FG, Weller JL, Møller M, Klein DC (2016) Melatonin synthesis: acetylserotonin O-methytransferase (ASMT) is strongly expressed in a subpopulation of pinealocytes in the male rat pineal gland. Endocrinology 157:2028–2040
4. Simonneaux V, Ribelayga C (2003) Generation of the melatonin endocrine message in mammals: a review of the complex regulation of melatonin synthesis by norepinephrine, peptides, and other pineal transmitters. Pharmacol Rev 55:325–395
5. Reiter RJ (1993) The melatonin rhythm: both a clock and a calendar. Experientia 49:654–664
6. King DP, Takahashi JS (2000) Molecular genetics of circadian rhythms in mammals. Annu Rev Neurosci 23:713–742
7. Vriend J, Reiter RJ (2015) Melatonin feedback on clock genes: a theory involving the proteasome. J Pineal Res 58:1–11
8. Torres-Farlan C, Seron-Ferre M, Dinet V, Korf H-W (2006) Immunocytochemical demonstration of day/night changes of clock gene protein levels in the murine adrenal gland: differences between melatonin-proficient (C3H) and melatonin-deficient (C57BL). J Pineal Res 40:64–70
9. Alonso-Vale MIC, Andreotti S, Mukai PY, Lima FHB (2008) Melatonin and the circadian entrainment of metabolic and hormonal activities in primary isolated adipocytes. J Pineal Res 45:422–429
10. Farias TSM, Chimin P, Oliveira AC, Amaral FG, Chimin P, Proença ARA, Leal FLT, Sertié RAL et al (2015) Pinealectomy interferes with the circadian clock genes expression in white adipose tissue. J Pineal Res 58:251–261
11. Acuña-Castroviejo D, Escames G, Venegas C, Diaz-Casado ME, Lima-Cabello E, López LC, Rosales-Corral S, Tan DX et al (2014) Extrapineal melatonin: sources, regulation, and potential functions. Cell Mol Life Sci 71:2997–3025
12. Sakaguchi K, Itoh MT, Takahashi N, Tamuri W, Ishizuka B (2013) The rat oocyte synthesizes melatonin. Reprod Fertil Dev 25:674–682

13. Coelho LA, Peres R, Amaral FG, Reiter RJ, Cipolla-Neto J (2015) Daily differential expression of melatonin-related genes and clock genes in rat cumulus-oocyte complex: Changes after pinealectomy. J Pineal Res 58:490–499
14. He C, Wang J, Zhang Z, Yang M, Li Y, Tian X, Ma T, Tao J et al (2016) Mitochondria synthesize melatonin to ameliorate its function and improve mice oocyte's quality under in vitro conditions. Int J Mol Sci 17:939. https://doi.org/10.3390/ijms17060939
15. Gonzalez-Arto M, Hamilton TRS, Gallego M, Gaspar-Torrubia E, Aguilar D, Serrano-Blesa E, Abecia JA, Pé-Pérez R et al (2016) Evidence of melatonin synthesis in the ram reproductive tract. Andrology 4:163–171
16. Tijmes M, Pedraza R, Valladares L (1996) Melatonin in the rat testis: evidence for local synthesis. Steroids 61:65–68
17. Itoh MT, Ishizuka B, Kuribayashi Y, Amemiya A, Sumi Y (1999) Melatonin, its precursors, and synthesizing enzyme activities in the human ovary. Mol Hum Reprod 5:402–408
18. Li C, Zhou X (2015) Melatonin and male reproduction. Clin Chim Acta 446:175–180
19. Frungieri MB, Mayerhofer A, Karina Zitta K, Pignataro OP, Calandra RS, Gonzalez-Calvar SI (2005) Direct effect of melatonin on Syrian hamster testes: melatonin subtype 1a receptors, inhibition of androgen production, and interaction with the local corticotropin-releasing hormone system. Endocrinology 146: 1541–1552
20. Qin F, Zhang J, Zan L, Guo W, Wang J, Chen L, Cao Y, Shen O et al (2015) Inhibitory effect of melatonin on testosterone synthesis is mediated via GATA-4/SF-1 transcription factors. RBM Online 31: 638–646
21. Reiter RJ, Rosales-Corral S, Tan DX, Jou MJ, Galano A, Xu B (2017) Melatonin as a mitochondria-targeted antioxidant: one of evolution's best ideas. Cell Mol Life Sci 74:3863–3881
22. Vera H, Tijmes M, Ronco AM, Valladares L (1993) Melatonin binding sites in interstitial cells from immature rat testes. Biol Res 26:337–340
23. Izzo G, Francesco A, Ferrara D, Campitiello MR, Serino I, Minucci S, d'Istria M (2010) Expression of melatonin (MT1, MT2) and melatonin-related receptors in the adult rat testes and during development. Zygote 18:257–264
24. Pozo D, Garcia-Mauriño S, Guerrero JM, Calvo JR (2004) mRNA expression of nuclear receptor RZR/RORα, melatonin membrane receptor MT_1 and hydroxindole-O-methytranferase in different populations of human immune cells. J Pineal Res 37:48–54
25. Smirnov AN (2001) Nuclear melatonin receptors. Biochemistry 66: 19–26
26. Venegas C, García JA, Doerrier HV, Volt H, Escames G, López LS, Reiter RJ, Acuña-Castroviejo D (2013) Analysis of the daily changes of melatonin receptors in the rat liver. J Pineal Res 54:313–321
27. Hoffman RA, Reiter RJ (1965) Rapid pinealectomy in hamsters and other small rodents. Anat Rec 53:19–22
28. Livak KJ, Schmittgen TD (2001) Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta CT}$ method. Methods 25:402–408
29. Vandesompele J, De Preter K, Pattyn F, Poppe B, Roy NV, De Paepe A, Speleman F (2002) Accurate normatization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. Genome Biol 3:RESEARCH0034
30. Nelson W, Tong YL, Lee JK, Halberg F (1979) Methods for cosinor-rhythmometry. Chronobiologia 6:305–323
31. Stefulj J, Hörtner M, Ghosh M, Schauenstein K, Rinner I, Wölfler A, Semmler J, Liebmann M (2001) Gene expression of the key enzymes of melatonin synthesis in extrapineal tissues of the rat. J Pineal Res 30:243–247
32. Luboshitzky R, Shen-Orr HP (2002) Seminal plasma melatonin and gonadal steroids concentrations in normal men. Arch Androl 48: 225–232
33. Rossi SP, Windschetti S, Matzkin ME, Terradas C, Ponzio R, Puigdomenech E, Levalle O, Calandra RS et al (2014) Melatonin in testes of infertile men: evidence for anti-proliferative and antioxidant effects on local macrophage and mast cell populations. Andrology 2:436–449
34. Rossi SP, Matzkin ME, Terradas C, Ponzio R, Puigdomenech E, Levalle O, Calandra RS, Frungieri MB (2012) New insights into melatonin/CRH signaling in the hamster Leydig cells. Gen Comp Endocrinol 178:153–163
35. Stocco DM, Clark BJ (1996) Regulation of the acute production of steroids in steroidogenic cells. Endocr Rev 17:221–244
36. Qin F, Zhang J, Cao H, Guo W, Chen L, Shen O, Sun J, Yi C et al (2014) Circadian alterations of reproductive functional markers in male rats exposed to 1800 Mhz radiofrequency field. Chronobiol Int 31:123–133
37. Liu X, Xu Y, Shi Q, Lu Q, Ma S, Xu X, Guo X (2013) Alterations of reproductive hormones and receptors of male rats at the winter and summer solstices and the effects of pinealectomy. Neuroendocrinol Lett 34:143–153
38. Stocco DM, Wang X, Jo Y, Manna PR (2005) Multiple signaling pathways regulating steroidogenesis and steroidogenic acute regulatory protein expression: more complicated than we thought. Mol Endocrinol 19:2647–2659
39. Wu C-S, Leu S-F, Yang H-Y, Huang B-M (2001) Melatonin inhibits the expression of steroidogenic acute regulatory protein and steroidogenesis in MA-10 cells. J Androl 22:245–254
40. González-Arto M, Vicente-Carrillo A, Martinez-Pastor F, Fernández-Alegre E, Roca J, Miró J, Rigau T, Rodríguez-Gil JE et al (2016) Melatonin receptors MT_1 and MT_2 are expressed in spermatozoa of several seasonal and nonseasonal breeder species. Theriogenology 86:1958–1968
41. Espino J, Ortiz A, Bejarano I, Lozano GM, Monllor F, García JF, Rodríguez AB, Pariente JA (2011) Melatonin protects human spermatozoa from apoptosis via melatonin receptor- and extracellular signal-regulated kinase-mediated pathways. Fertil Steril 95:2290–2296
42. Dubocovich ML, Yun K, Al-Ghoul WM, Benloucif S, Masana MI (1998) Selective MT2 melatonin receptor antagonists block melatonin-mediated phase advances of circadian rhythms. FASEB J 12:1211–1220
43. Nakajima Y, Ikeda M, Kimura T, Honma S, Ohmiya Y, Honma K (2004) Bidirectional role of orphan nuclear receptor RORα in clock gene transcriptions demonstrated by a novel reporter assay system. FEBS Lett 565:122–126
44. Kennaway DJ, Boden MJ, Varcoe TJ (2012) Circadian rhythms and fertility. Mol Cell Endocrinol 349:56–61
45. Alvarez JD, Hansen A, Ord T, Bebas P, Chappell PE, Giebultowicz JM, Williams C, Moss S et al (2008) The circadian clock protein BMAL1 is necessary for fertility and proper testosterone production in mice. J Biol Rhythm 23:26–36
46. Reiter RJ (1968) The pineal gland and gonadal development in male rats and hamsters. Fertil Steril 19:1009–1017
47. Majrashi KAA, Barakat IAH, Al-Himaidi AR, Adham KG (2017) Effects of exogenous melatonin treatment on the reproductive characteristics and progeny of male rats exposed to different periods from light and darkness. Physiol Res 66:507–518

Publisher's Note Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.