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Melatonin replacement restores the circadian behavior in adult rat Leydig cells after pinealectomy

Aleksandar Z. Baburski, Srdjan J. Sokanovic, Marija M. Janjic, Natasa J. Stojkov-Mimic, Maja M. Bjelic, Silvana A. Andric, Tatjana S. Kostic*

Department of Biology and Ecology, Faculty of Sciences, University of Novi Sad, Novi Sad, Serbia

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ABSTRACT

Melatonin actions on oscillators in reproductive organs are poorly understood. Here we analyzed melatonin effects on rhythmic expression of clock and steroidogenesis-related genes in adult rat Leydig cells (LCs). The effect of melatonin was tested both *in vivo* using pinealectomized and melatonin-substituted rats and *in vitro* on isolated LCs. Data revealed 24-h-rhythmic expression of clock genes (*Bmal1, Per1,2,3, Rev-erba,b, Rorb*), steroidogenic genes (*Star, Cyp11a1, Cyp17a1*), and genes of steroidogenic regulators (positive-*Nur77*, negative-*Arr19*). Pinealectomy increased 24-h-oscillations of serum testosterone and LC's cAMP levels, expression of *Insl3, Per1*, *Star*/StAR, *Hsd3b1/2*, *Nur77*, decreased *Arr19* and canceled *Per2* oscillatory expression pattern. At hypothalamic-pituitary level, pinealectomy increased mesor of *Gnrh, Lhb* and rhythm robustness of *Mntr1a* expression. All parameters disturbed were restored by melatonin-replacement. *In vitro* studies did not confirm direct melatonin effects on neither clock nor steroidogenic genes. Accordingly, melatonin influence 24-h-rhythmic LC-function likely through hypothalamic-pituitary axis and consequently cAMP-signaling in LCs.

1. Introduction

Physiological rhythms entrained by the circadian clock are present in virtually all organs including those of the reproductive system (Albrecht, 2012). In mammals, circadian timing is driven by a central rhythm generator located in the suprachiasmatic nucleus (SCN) of the hypothalamus that receive external or internal signals, modify them, and synchronize all the other peripheral clocks by conveying timing information (Welsh et al., 2010). At the molecular level, circadian rhythms are driven by interlocked loops of clock genes coding for various transcription factors: CLOCK/BMAL1 being the positive part of the loop, PER/CRY being the negative part of the loop, and both being modulated by other stimulatory (RORα) or inhibitory (REV-ERBα/β) transcription factors (Albercht and Eichele, 2003; Ko and Takahashi, 2006). These clock genes generate endogenous oscillations that drive rhythmic expression of downstream genes and thus physiological processes.

Evidence indicates that the circadian clock regulates the serum concentrations of many reproductive hormones and that the primary clock genes, *Clock* and *Bmal1*, strongly influence reproductive competency (Alvarez et al., 2008; Liang et al., 2013; Hodžić et al., 2013). In males, testosterone is pivotal androgenic hormone that controls fertility, development and maintenance of the male

Abbreviations: ACTB, protein β-actin; *Adcy*, gene for adenylyl cyclases; *Bmal1*, brain-muscle Arnt-like protein 1; BSA, bovine serum albumin; cAMP, cyclic adenosine monophosphate; *Cga*, alpha polypeptide of glycoprotein hormones; *Ck1*, gene for casein kinase 1; Clock/CLOCK, gene/protein for circadian locomotor output cycles kaput; CRE, cAMP-responsive element; *Creb*/CREB, gene/protein for cAMP response element-binding protein; *Cry*/CRY, gene/protein for cryptochrome; *Cyp11a*/CYP11a, gene/protein for cytochrome P450 side chain cleavage enzyme; *Cyp17a*/CYP17a, gene/protein for 17α-hydroxylase/C17-20 lyase; *Dax1*/DAX1, gene/protein for dosage-sensitive sex reversal; DHT, dihydrotestosterone; DMEM/F12, Dulbecco's modified eagle medium; *E4bp4*, gene for Nuclear factor interleukin 3 regulated; *Gapdh*, gene for glyceraldehyde 3-phosphate dehydrogenase; *Hsd17b*, gene for hydroxysteroid dehydrogenase 17β; *Hsd3b*, gene for hydroxysteroid dehydrogenase 3β; *Insl3*/INSL3, gene/protein for insulin-like 3; LCs, Leydig cells; LH, luteinizing hormone; *Lhr*/LHR, gene/protein for luteinizing hormone receptor; *Lhb*, gene for LH β subunit; *Mntr1a,b*/MNTR1a,b, gene/protein for melatonin receptor 1 or 2; *Npas2*, gene for Neuronal PAS domain-containing protein 2; *Nur77*, gene for nerve growth factor; P, pinealectomized rats; P + M, pinealectomized rats received melatonin; *Pde*, cAMP-specific 3′,5′-cyclic phosphodiesterase; *Per1*, period circadian protein 1; *Per2*, period circadian protein 2; PKA, cAMP dependent protein kinase; RIA, radioimmunoassay; *Rev-erba/b* / REV-ERBA/B, gene/protein for reverse viral erythroblastis oncogene product alpha/beta; *Ror*/ROR, retinoid acid related orphan receptor; RQ-PCR, relative quantification polymerase chain reaction; *Sf1*, gene for steroidogenic factor 1; SP, sham pinealectomized rats; *Star*/StAR, gene/protein for steroidogenic acute regulatory protein; T, testosterone; ZT, Zeitgeber (German: "time giver") time.

* Corresponding author. Department of Biology and Ecology, Faculty of Sciences, University of Novi Sad, Dositeja Obradovica Sq 2, 21000 Novi Sad, Serbia.
E-mail address: tatjana.kostic@dbe.uns.ac.rs (T.S. Kostic).

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phenotype including the reproductive system, but also muscle strength, cognition and sexual function (Basaria, 2013). It is secreted from Leydig cells (LCs) in response to pituitary gonadotropin luteinizing hormone (LH) (Dufau, 1998; Haider, 2004) and consequent cAMP dependent activation and expression of proteins involved in steroidogenesis such as steroidogenic acute regulatory protein (*Star*/StAR), cytochrome P450 side chain cleavage enzyme (*Cyp11a1*/CYP11A), hydroxysteroid dehydrogenase 3β (*Hsd3b*/HSD3B), 17α-hydroxylase/C17-20 lyase (*Cyp17a*/CYP17A) and hydroxysteroid dehydrogenase 17β (*Hsd17b*/HSD17B) (Payne and Hales, 2004). Testosterone secretion from LCs has long been recognized to have a low-amplitude of diurnal rhythm (Mock et al., 1978; Chacon et al., 2004). Still, integral network of signals linking the SCN and peripheral oscillators including gonads is presently not well understood.

Some of the SCN clock outputs are hormones of the pineal gland especially melatonin whose primary function is to transduce light and dark information to whole body physiology (Arendt, 2005). Melatonin circadian synthesis plays a crucial role in the regulation of circadian and seasonal changes in various aspects of physiology and neuroendocrine functions. Pineal-derived melatonin, acting through the SCN and the pars tuberalis, influences the synthesis and release of the hypothalamic GnRH, therefore affecting the secretion of pituitary gonadotropin hormones and eventually, the testicular function (Pevet and Challet, 2011). Melatonin acts through specific G-protein-coupled membrane receptors (MNTR1A, MNTR1B), cytosolic quinone reductase 2, previously known as melatonin receptor 3 (Slominski et al., 2012) and putative nuclear RZR/ROR retinoid receptors (Carrillo-Vico et al., 2005). In males, melatonin exerts its negative effects on the testis through specific high affinity receptors MNTR1A and MNTR1B in the hypothalamic-pituitary-testicular axis (Yilmaz et al., 2000; Gillette and McArthur, 1996; Valenti et al., 1997). Although melatonin rhythmicity is undoubtedly dependent upon central SCN clock control (Reiter et al., 2011), involvement of melatonin in peripheral clock control especially in reproductive organs is unclear. Further, the time-giver role of melatonin and its involvement in temporal organization of LCs steroidogenesis was not studied before.

Here we hypothesize that melatonin alters the rhythmic activity of LCs with consequences on the testosterone production. Therefore, we attempted to characterize rhythmic activity of LCs and determine whether it is affected by melatonin. To do that, we utilized *in vivo* and *in vitro* experimental approaches. Results showed involvement of melatonin in modulation of rhythm of LCs clock and steroidogenic elements expression but this effect is likely achieved through hypothalamic-pituitary axis.

2. Material and methods

2.1. Ethical approval

All the experimental protocols were approved by the Ethical Committee on Animal Care and Use at the University of Novi Sad (statement no. I-2011-02), operating under the rules of National Council for Animal Welfare and following statements of National Law for Animal Welfare (copyright March 2009). All our experiments were performed and conducted in accordance with the National Research Council (NRC) publication Guide for the Care and Use of Laboratory Animals (National Academy of Sciences, Washington DC, 1996) and NIH Guide for the Care and Use of Laboratory Animals (NIH Publications, No. 80 23, revised, 7th ed., 1996). All the experiments were carried out in the Laboratory for Reproductive Endocrinology and Signaling, DBE, Faculty of Sciences at University of Novi Sad.

2.2. Animals

Male *Wistar* rats bred and raised in the animal facility of the Faculty of Sciences, University of Novi Sad (Novi Sad, Serbia) were used for the experiments. Animals were raised in controlled environmental conditions (22 ± 2 °C; 12 h light − 12 h dark cycle, lights on at 6:00 AM) and provided with food and water *ad libitum*. The 70-day-old male rats were randomized into 3 groups: (1) pinealectomized rats (P); (2) pinealectomized rats received melatonin (4 mg/kg; Now Foods, Bloomingdale, IL) dissolved in pure distillated water by oral dosing (P + M); (3) sham pinealectomized rats (SP). Rats were fed with melatonin or distillated water 1 h before light was turned off, every day for one month. After one month animals were sacrificed in six time points during the 24 h (ZT0, ZT5, ZT11, ZT16, ZT20 and ZT24, ZT0 − moment when light was turned on), 3–5 animals per time point for each group. Rats were quickly decapitated without anesthesia (to avoid the effect of anesthesia on serum hormone levels), and the trunk blood was collected. Individual serum samples were stored at −80 °C until hormone assay was performed. Free-hand dissection of hypothalamus covered ventrolateral area between optic chiasma and mammillary bodies was performed. Hypothalamus and pituitary were isolated and rapidly buried in dry ice and stored at −80 °C until RNA isolation were performed. *In vivo* experiments were repeated three times.

2.3. Pinealectomy

The animals were anesthetized intraperitoneally with 50 mg/kg of Thiopentone sodium (CIRON Drugs & Pharmaceuticals) after which the ablation of the pineal gland was performed by method of Hoffman and Reiter (1965) with some modifications. The same procedure was performed on the SP animals but the pineal gland was not taken out. The SP rats were used as control. Pinealectomy was verified by post-mortem morphological analysis in addition to serum melatonin measurement.

2.4. Hormones and cyclic AMP measurements

Androgen level was measured by RIA and it was referred to as testosterone + dihydro-testosterone (T + DHT) because the anti-testosterone serum No. 250 showed 100% cross-reactivity with DHT (Andric et al., 2007; Kostic et al., 2010). All samples were measured in duplicate in one assay (sensitivity: 6 pg per tube; intra-assay coefficient of variation 5–8%). Androgen level in serum was assayed in 100 μL by direct RIA (without extraction). Melatonin was measured in 100 μL of serum using Rat Melatonin Elisa Kit (MyBioSource, Cat. No. MBS267866) (sensitivity: 5 pg/mL; intra-assay coefficient of variation ≤8%). Cyclic AMP was extracted from cell content by ethanol using a procedure described previously (Kostic et al., 2001). Level of intracellular cAMP was measured from Leydig cell's content by the cAMP EIA Kit (Cayman, Ann Arbor, MI) with a limit of quantification of 0.1 pmol/mL for acetylated samples.

2.5. Leydig cells purification

For gene expression analysis we used LCs purified from testes obtained from P, P + M and SP rats prepared as described previously by our group (Andric et al., 2007; Kostic et al., 2010). Briefly, testes were quickly removed, decapsulated, placed in medium 199 containing 0.25 mg/mL collagenase, 1.5% BSA, 20 mM HEPES, sodium bicarbonate and antibiotics and incubated in a shaking water bath oscillating at 120 cycles/min at 34 °C for 15 min. The dissociated cells, were filtered through mesh no. 100 (Sigma) and the cell suspension was centrifuged twice at 160 × g for 5 min at room