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Original paper

Circadian rhythm patterns of NO-cGMP signaling are moderately synchronized by melatonin in testosterone-producing Leydig cells

Aleksandar Z Baburski, Marija LJ Medar, Silvana A Andric, Tatjana S Kostic[1]

University of Novi Sad, Faculty of Sciences, Department of Biology and Ecology, Trg Dositeja Obradovića 2, Novi Sad, Serbia

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Summary. It is established that pineal melatonin is involved in circadian regulation of testosterone secretion from Leydig cells. However, the precise routes of this regulatory involvement are still unknown. As cGMP is also regarded as modulator of steroidogenesis, we sought to study the effects of melatonin on cGMP variations and the expression of genes that encode elements of NO-cGMP signaling pathways in Leydig cells from adult rats. The melatonin effect was tested *in vivo* on pinealectomized and melatonin-replaced rats and *ex vivo* on primary culture of Leydig cells. Pinealectomy increased amplitude of serum testosterone circadian oscillation which was restored by melatonin treatment. Real-time quantitative PCR analysis revealed a circadian transcriptional pattern of *Nos2* (gene encoding NO producer) and *Pde5a* (gene encoding cGMP remover) in Leydig cells. Pinealectomy increased transcription of *Nos3* and *Pde5a*in certain time points (ZT11 and ZT5, respectively). Altered patterns of gene transcription were restored by melatonin-replacement. The transcription of *Gucy1a3*, *Gucy1b3* (genes encoding subunits of cGMP producer) and *Prkg1* (gene encoding the main effector of NO-cGMP signaling pathway) was not affected by pineal abolition. Although, pinealectomy affected expression of some genes of NO-cGMP signaling, the circadian variation of cGMP in Leydig cells was not significantly changed. *Ex vivo* studies revealed that the effect of melatonin on nitrite and testosterone production in Leydig cells is not direct but most probably mediated through the reproductive axis. Altogether, obtained results revealed circadian rhythm of NO-cGMP signaling in rat Leydig cells which is slightly synchronized by melatonin.

Keywords: cGMP, circadian rhythm, Leydig cell, melatonin, pineal.

INTRODUCTION

The physiology of Leydig cells displays a circadian rhythm driven by a complex interaction of reproductive axis hormones and the circadian system. The final output of this regulatory process is a circadian pattern of steroidogenic genes expression and testosterone production (Baburski et al. 2015, 2016). Although Leydig cells possess their own timing system (Baburski et al. 2015, 2016), circadian timing is driven by a central rhythm generator located in the suprachiasmatic nucleus (SCN) of the hypothalamus. SCN receive external or internal signals, modify them, and synchronize all other peripheral clocks by conveying timing information (Welsh et al. 2010). One 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). This hormone plays a crucial role in the regulation of circadian and seasonal changes in various aspects of physiology and neuroendocrine functions. Illustrating this, intact pineal is crucial for a seasonal testis regression and inhibition of sexual behavior during the short photoperiod (Prendergast et al. 2009; Yilmaz et al. 2000). Although it is known that in rats pinealectomy increases amplitude of circadian oscillation of serum testosterone (Baburski et al. 2015) the precise routes of this regulatory involvement in Leydig cells is still unknown. As we showed earlier (Baburski et al. 2015), one regulatory pathway through which melatonin may affect testosterone production includes cAMP signaling, that is consequently activated by pituitary gonadotropin lutein-

*Corresponding author, e-mail: e-mail: tatjana.kostic@dbe.uns.ac.rs

izing hormone (LH) (Dufau 1998; Haider 2007). Activation of cAMP signaling events facilitates cholesterol mobilization and transportation into mitochondria where steroidogenesis begins (Payne et al. 2004).

NO-cGMP signaling is also involved in regulation of Leydig cells function (Andric et al. 2007)and its activation modulates testicular steroidogenesis in accord with systemic hormones and locally produced NO and cGMP (Davidoff et al. 1995; Davidoff et al. 1997; Tatsumi et al. 1997; Weissman et al. 2005; Andric et al. 2007). In Leydig cells, NO is generated by endothelial NO synthases (NOS3) and inducible NO synthases (NOS2) (Andric et al. 2010a; 2010b). Stimulation of cGMP production either by nitric oxide (NO)-dependent soluble (GUCY1) or membranous-bound guanylyl cyclases activates protein kinase G (PRKG) that could facilitate cholesterol transport into mitochondria and augment steroidogenesis (Gambaryan et al. 2003; Andric et al. 2007). The cGMP and cAMP signaling pathways act synergistically and both phosphorylate StAR protein to enhance testosterone production(Andric et al. 2010b). The cGMP signal is terminated by PDE5 action (Andric et al. 2010a, 2010b).

In the present study, we wanted to enrich our previous work by analyzing the effects of melatonin on the circadian pattern of cGMP variations as well as the expression of genes that encode elements of NO-cGMP signaling pathway in Leydig cells using both an *in vivo* and *ex vivo* approach.

MATERIAL AND METHODS

Ethical approval

Experiments were approved by the Ethics Committee on Animal Care and Use at the University of Novi Sad (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 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.

Animals

Male *Wistar* rats used for experiments were bred and raised in the animal facility of the Faculty of Sciences, University of Novi Sad (Novi Sad, Serbia) under controlled environmental conditions (22 ± 2 °C; 12 h light - 12 h dark cycle, lights on at 6:00 AM) providing with water and food *ad libitum*. Experiments were designed as previously described (Baburski et al. 2015). Briefly, 3 groups were formed from 70-day-old male Wistar rats: (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). Animals were fed with melatonin or distillated water 1 h before light was turned off, every day for one month, after which were sacrificed in six time points during the 24 hours (ZT0, ZT5, ZT11, ZT16, ZT20 and ZT24, ZT0 - moment when light was turned on), 4 animals per time point for each group. After decapitation, trunk blood was collected (anesthesia was not used because of potential effects on serum hormone levels). Individual serum samples were stored at -80 °C until hormone assay was performed. *In vivo* and *ex vitro* experiments were repeated three times.

Pinealectomy

As described previously (Baburski et al. 2015) animals were anesthetized intraperitoneally with 50 mg/kg of Thiopentone sodium (CIRON Drugs & Pharmaceuticals) followed by pinealablation performed by the method of Hoffman and Reiter (1965) with some modifications (Kostic et al. 1997; Baburski et al. 2015). SP rats (control) were treated using the same procedure except that the pineal gland was not taken out. Pinealectomy was verified by serum melatonin measurement and post-mortem morphological analysis.

Hormones, cyclic GMP, and nitrite measurements

Androgen levels, referred to as testosterone + dihydrotestosterone (T + DHT), were measured by direct RIA (without extraction) using anti-testosterone serum No. 250 (Kostic et al. 2010; Baburski et al. 2015). All samples were measured in duplicate in one assay (sensitivity: 6 pg per tube; intra-assay coefficient of variation 5–8%). 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 GMP was extracted from cell content by ethanol following an earlier described procedure (Kostic et al. 2001). cGMP levels were measured using a cGMP EIA kit (Cayman Chemicals), with a limit of quantification of 0.07 pmol/ml (at 80% B/B0) for acetylated cGMP. Concentration of nitrite, a stabile metabolite of NO, was measured in culture medium. Samples were mixed with an equal volume of Griess reagent and the absorbance was measured at 546 nm (Sokanovic et al. 2013).

Leydig cells purification

Leydig cells were isolated from control and experimental Wistar rats using Percoll gradients as previously described by our group (Baburski et al. 2015). Staining for HSD3B activity was used for determination of the proportion of Leydig cells in primary culture. Viability was measured using the

0.2% Trypan blue dye exclusion test (Sigma Inc.) (Baburski et al. 2015) showing viability higher than 90%.

Ex vivo experiments

To examine direct effect of melatonin on testosterone and nitrite production, primary Leydig cell culture was isolated from control rats and *ex vivo* experiments were performed. Leydig cells were plated (3 x 10^6 cells in 55 mm dish), and after a recovery period in DMEM/F12-10% FBS, the culture medium was changed with serum-free DMEM/F12 (referred to as ZT0). At ZT0 and ZT9 cells were stimulated with/without melatonin (1 μM; Sigma, St Louis, MO) in the presence/absence of hCG (human horionic gonadotropin; 50 ng/ml) for 3 h *i.e.* ZT0-ZT3 and ZT9-ZT12. After stimulation culture media was collected for androgen and nitrite measurement. *Ex vivo* experiments were repeated three times.

RNA isolation and cDNA synthesis

Total RNA from purified Leydig cells was isolated with the Rneasy kit reagent following the protocol recommended by the manufacturer (QIAGEN, Valencia, California). After DNase I treatment, first-strand cDNA was synthesized according to the manufacturer's instructions as previously described (Invitrogen, Carlsbad, California) (Kostic et al. 2010; Baburski et al. 2015).

Real-time PCR and relative quantification

Relative gene expression was quantified by RQPCR using SYBR green-based chemistry (Applied Biosystems, Foster City, California) in the presence of a 5 μl aliquot of the reverse transcription reaction product (25 ng RNA calculated based on the starting RNA) and specific primers. The primer sequences used for RQPCR analysis and GenBank accession codes (www.ncbi.nlm.nih.gov/sites/entrez) are provided in Supplemental Tab 1. *Gapdh* was used to correct for variations in RNA content, and also served as an endogenous control. The relative quantification of each gene was performed in duplicate for each experiment.

Statistical and rhythm analysis

The results represent group means ± SEM values of three *in vivo* or *ex vivo* experiments. Rhythm parameters (p, Robustness, MESOR, Amplitude and Acrophase) were obtained by the cosinor method using the program Cosinor, with data fit to a 24 h period (http://www.circadian.org/softwar.html). The statistical significance ($p < 0.05$) between treated (P, P+M) groups and control (SP) within the same time point was analyzed using the Mann Whitney test.

RESULTS

Pinealectomy changed the circadian profile of serum melatonin and testosterone levels

As expected, the level of melatonin in serum showed circadian fluctuations with a peak at the dark phase (around ZT19). The rise of serum melatonin during the night was prevented by pinealectomy and melatonin replacement increased its levels (Fig. 1A, for properties of circadian rhythm please see Table 1).

Serum testosterone showed a low-amplitude diurnal rhythm and reached a peak at the beginning of the dark phase (ZT13) and nadir in ZT1 (Fig. 1B, Table 1). Pineal removal increased the amplitude and slightly advanced the peak (SP around ZT 13 h vs. P around ZT 11 h 40 min) of testosterone oscillations (Fig. 1B, Table 1).

Effect of pinealectomy on cGMP circadian homeostasis and expression patterns of NO-cGMP related genes

In an attempt to detect the effect of melatonin on cGMP oscillations, the 24-h fluctuation of its content in Leydig cells from all three groups of rats was measured. Circadian rhythmicity was detected in all examined groups with an acrophase around ZT8 (Fig. 2A, Table 1). However, no significant difference in rhythm parameters of cGMP circadian variations was observed among the groups.

Following analysis of gene expression in NO producers, an elevation in transcription of *Nos3* in pinealectomy at ZT11 was observed, however *Nos3* as well as *Nos1* did not display any rhythmicity (Fig. 2B, Table 1). On the other hand, *Nos2* displayed an oscillatory pattern in transcription with advanced acrophase in pinealectomy (ZT6 for SP and ZT3 for P) (Fig. 2B, Table 1).

Transcription of *Gucy1a3* and *Gucy1b3*, genes responsible for cGMP production, were not cyclic, and no significant changes in transcription levels were observed (Fig. 2C, Table 1). *Pde5a*, a gene involved in cGMP degradation, showed daily oscillations with a peak around ZT6, including elevated transcription in ZT5 in group P (Fig. 2E, Table 1).

Expression of *Prkg1* gene, the main effector in the NO-cGMP signaling pathway, showed no rhythmicity and was not affected with pineal abolition (Fig. 2D, Table 1). We were not able to detect a significant amount of *Prkg2* mRNA in adult Leydig cells.

Melatonin does not act directly on testosterone and nitrite production in Leydig cells

To examine the direct effect of melatonin on testosterone and nitrite production isolated Leydig cells were challenged with melatonin and an analog of the LH receptor

Table 1. Rhythm parameters in adult rat Leydig cells (period fit to 24h). Pinealectomized (P), sham pinealectomized (SP) and melatonin-substituted (P+M) rats were euthanized at six time points during a period of24h (ZT0, ZT5, ZT11, ZT16, ZT20 and ZT24) and serum was collected for determination of melatonin and testosterone levels. Leydig cells from different time points were purified and used for cGMP measurements and mRNA isolation followed by qPCR analysis of gene expression. Data are group means of three experiments; SEM values were in range of 10-20% of mean values. Presented data were obtained by the Cosinor method.

	Group	Robustness	p	Mesor	Amplitude	Acrophase (ZT)
Melatonin	SP	67.7%	0.000071	33.9858	20.9563	283° / 18 h 53 min
	P	0.0%	0.558851			
	P+M	31.8%	0.008509			
Testosterone	SP	47.9%	0.000611	1.5638	0.7912	194° / 12 h 56 min
	P	71.3%	0.000013	2.2608	1.5497	177° / 11 h 46 min
	P+M	69.1%	0.000148	1.3573	0.7546	201° / 13 h 23 min
cGMP	SP	66.1%	0.000501	2.3712	0.9509	124° / 08 h17 min
	P	70.5%	0.000234	2.5758	1.0648	114° / 07 h36 min
	P+M	65.8%	0.000519	2.8869	1.0298	125° / 08 h19 min
Nos1	SP	0.1%	0.991262			
	P	11.5%	0.599183			
	P+M	7.3%	0.702030			
Nos2	SP	57.9%	0.001821	1.0449	0.4727	92° / 06 h 06 min
	P	36.7%	0.031849	0.8218	0.2962	41° / 02 h 44 min
	P+M	37.4%	0.029094	0.8522	0.2681	355° / 23 h 40 min
Nos3	SP	6.3%	0.504123			
	P	14.1%	0.319417			
	P+M	5.8%	0.635438			
Gucy1a3	SP	10.1%	0.401996			
	P	17.6%	0.214090			
	P+M	9.3%	0.583125			
Gucy1b3	SP	6.3%	0.617626			
	P	16.8%	0.319145			
	P+M	3.8%	0.752817			
Prkg1	SP	0.8%	0.937991			
	P	9.4%	0.517696			
	P+M	2.3%	0.782653			
Pde5a	SP	59.7%	0.001385	0.8942	0.4333	87° / 05 h 46 min
	P	70.1%	0.000251	1.1267	0.7378	110° / 07 h 19 min
	P+M	65.2%	0.000576	0.8955	0.5014	95° / 06 h 19 min

ZT is Zeitgeber time; **p** *value* - probability of outcome.

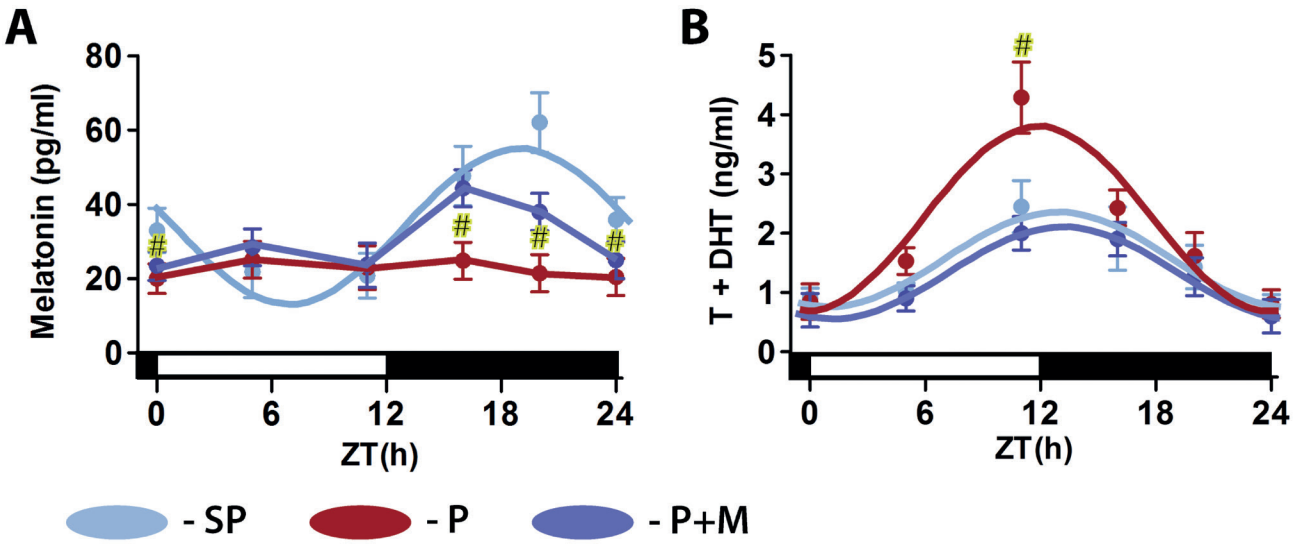

Fig. 1. Pinealectomy influenced circadian rhythm of serum melatonin and testosterone. SP, P and P+M rats were euthanized in six time points over a period of 24h (ZT0, ZT5, ZT11, ZT16, ZT20 and ZT24) and serum was collected for determination of melatonin (**A**) and androgen (T+DHT) (**B**) levels. Data points are group mean ± SEM values, n=12. In this and the following figures, best fit curves werecomposed using theCosinor method. For rhythm parameters (Robustness, MESOR, Amplitude and Acrophase) see Suppl. Table 1. **#** Statistical significance between control (SP) and experimental groups (P, P+M) for the same time point ($p < 0.05$). In this and the following figures ZTstands for Zeitgeber time.

(hCG) alone or in combination. Melatonin did not influence testosterone or nitrite production in basal or hCG-supported conditions (Fig. 3) supporting the absence of a direct effect of melatonin on Leydig cells.

DISCUSSION

Results from this study support the circadian nature of rat Leydig cell endocrine activity, including the circadian rhythm of NO-cGMP signaling. This is suggested by the presence of a circadian pattern in the transcription of *Nos2,* and *Pde5* as well as the circadian rhythm of cGMP synthesis in Leydig cells of adult rats. The circadian patterns observed in NO-cGMP signaling suggest its possible involvement in regulation of the circadian endocrine function of Leydig cells. This is supported by the fact that the NO-cGMP signaling system has the capacity to modulate steroidogenesis: NO at higher concentrations directly suppresses Leydig cell function by inhibiting heme containing steroidogenic enzymes, such as CYP11A1 and CYP17A1 (Del Punta et al. 1996; Drewett et al. 2002); while at low concentrations, NO stimulates Leydig cell steroidogenesis by activating GUCY1-cGMP-PRKG1 signaling (Lu et al. 1993; Valenti et al. 1999; Andric et al. 2007; Andric et al. 2010a; Andric et al. 2010b) and subsequent phosphorylation of StAR protein (Gambarayan et al. 2003; Andric et al. 2007, 2010a).

However, the mechanism regulating synchronization of adult Leydig cell rhythm is still unclear and could involve pineal hormones which likely modulate steroidogenesis via nightly synthesis and secretion. Results from our study support a modulatory role for pineal hormones on Leydig cell circadian rhythm, since pineal removal was observed in the present study to increase and advance the peak of testosterone secretion. Pineal regulation may occur through indirect influence of the main pineal hormone, melatonin, on the SCN (Lu et al. 1993; Starkey et al. 1995), or may result from a direct negative action on the reproductive axis (Reiter 1991; Baburski et al. 2015) or even on Leydig cells (Valenti et al. 1999). Earlier results suggest melatonin influence on the circadian function of Leydig cells from adult rats, most likely through the hypothalamic-pituitary axis and consequently cAMP-signaling and *Star*/StAR expression (Baburski et al. 2015).

There is also evidence that melatonin acts via the NO-cGMP signaling pathway. Most data indicate that melatonin has an inhibitory effect on the NO-cGMP signal transduction cascade. Several studies have revealed an inhibitory effect for melatonin on NO concentrations (Silva et al. 2007; Valero et al. 2006), NO synthase activity (Saenz et al. 2002; Esposito et al. 2008) or NO synthase expression levels (Chang et al. 2008). Melatonin is known to reduce guanylate cyclase activity and cGMP concentrations (Tamura et al. 2006), reduces the expression levels of GUCY mRNA (Strumpf et al. 2009), and increases the activity of cGMP-specific phosphodiesterases (Shukla et al. 2012) in several mammals, tissues, and cell lines.

Results obtained from our *in vivo* study suggest a stimulatory effect of pinealectomy on the transcriptional patterns

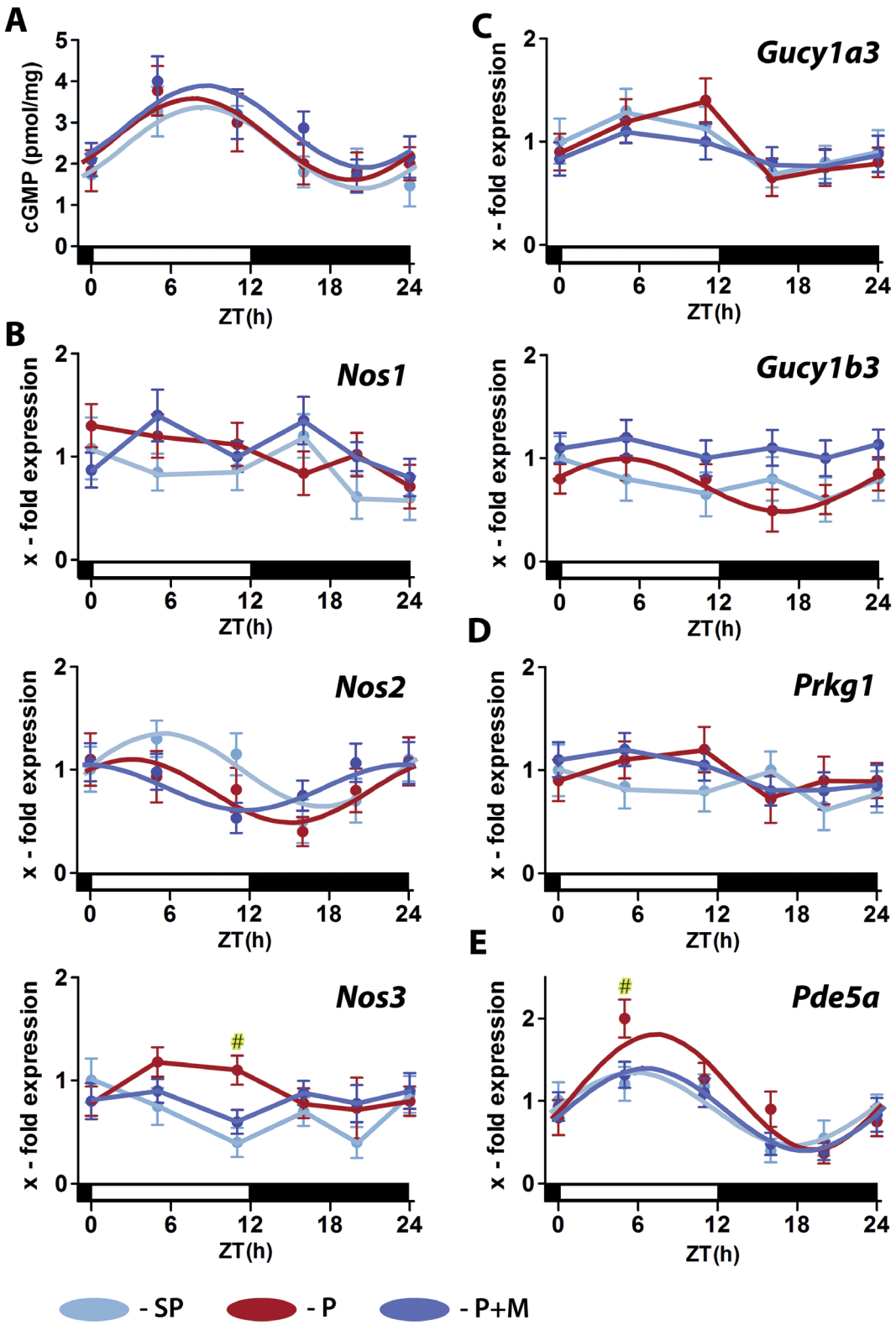

Fig. 2. Effect of pinealectomy on cGMP circadian homeostasis in rat Leydig cells. Leydig cells obtained from SP, P and P+M rats were isolated at different time points and intracellular content was collected for cGMP measurements (**A**) and used as a source of mRNA for RQPCR in the presence of primers specific for *Nos1, Nos2, Nos3* (**B**) *Gucy1a3* and *Gucy1b3* (**C**), *Prkg1* (**D**) and *Pde5a* (**E**). Data points are group mean ± SEM values, n = 3. For rhythm parameters see Suppl. Table 1. **#** Statistical significance between control (SP) and experimental groups (P, P+M) for the same time point ($p < 0.05$).

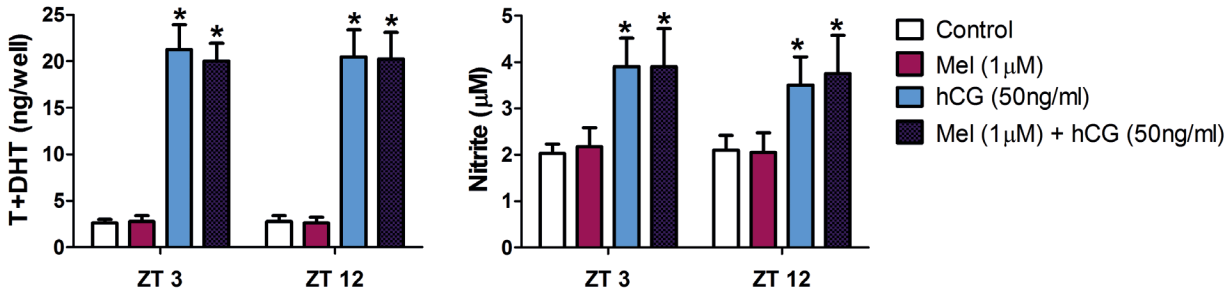

Fig. 3. *Ex vivo* effects of melatonin on testosterone and nitrite production in rat Leydig cells. To examine the direct effect of melatonin on testosterone and nitrite production, Leydig Cells were isolated from adult rat testes and plated (3 x 10^6 cells), and after a recovery period in DMEM/F12-10% FBS, the culture medium was changed with serum-free DMEM/F12 (referred to as time ZT0). At ZT0 and ZT9 cells were stimulated with or without melatonin (1 μM) in the presence or absence of hCG (50 ng/ml) for 3 h (i.e. ZT0-ZT3 and ZT9-ZT12). After stimulation, culture media was collected for androgen and nitrite measurements. Data points are group mean ± SEM values of three independent experiments.

of *Nos3* and *Pde5a* in Leydig cells. However, circadian variations of intracellular cGMP levels remained unchanged. This supports the possibility that transcriptional changes of *Nos3* and *Pde5a* might cancel each other's effects on cGMP levels in the Leydig cells of pinealectomized rats. The observed rise in the amplitude of testosterone production associated with pinealectomy is probably mediated through the reproductive axis and cAMP signaling pathways (Baburski et al. 2015). However, further studies based on these data are needed to better define the relationship between pineal, cGMP signaling and the circadian rhythm of testosterone production.

Our *ex vivo* experiments using isolated Leydig cell primary culture suggest that melatonin has no direct effect on testosterone production under either basal or hCG-stimulated conditions. Furthermore, we were unable to detect significant transcript levels for *Mntr1a* and *Mntr1b* (melatonin receptors) in the Eliding cells of adult rats (Baburski et al. 2015). This may be connected to the observation that melatonin receptor expression undergoes profound developmental changes resulting in low widespread receptor distribution in adults (Davis 1997).

Many studies have shown that melatonin and its metabolic derivatives possess strong free radical scavenging properties (Reiter et al. 2001). These metabolites are potent antioxidants against both ROS (reactive oxygen species) and RNS (reactive nitrogen species). The mechanisms by which melatonin and its metabolites protect against free radicals and oxidative stress include direct scavenging of radicals and radical products, induction of the expression of antioxidant enzymes, reduction of the activation of pro-oxidant enzymes, and maintenance of mitochondrial homeostasis (Acuna-Castroviejo et al. 2001). However, in our *ex vivo* experiments we did not observe changes in the levels of nitrite (a stabile metabolic product of NO) in Leydig cells, associated with melatonin under either basal or hCG stimulated conditions.

CONCLUSION

NO-cGMP signaling in testosterone-producing Leydig cells displays a circadian rhythm which is modulated by melatonin; most likely through the reproductive axis. Further studies are necessary to unravel the complex interactions of reproductive axis hormones and pineal hormones on circadian signaling that govern testosterone production.

Acknowledgments

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REFERENCES

Acuña-Castroviejo D, Martín M, Macías M, Escames G, León J, Khaldy H, Reiter RJ. 2001. Melatonin, mitochondria, and cellular bioenergetics. Journal of Pineal Research. 30:65–74.

Andric SA, Janjic MM, Stojkov NJ, Kostic TS. 2007. Protein kinase G-mediated stimulation of basal Leydig cell steroidogenesis. American Journal of Physiology. Endocrinology and Metabolism. 293:E1399–E1408.

Andric SA, Janjic MM, Stojkov NJ, Kostic TS. 2010a. Sildenafil treatment in vivo stimulates Leydig cell steroidogenesis via the cAMP/cGMP signaling pathway. American journal of physiology. Endocrinology and metabolism. 299:E544–E550.

Andric SA, Janjic MM, Stojkov NJ, Kostic TS. 2010b. Testosterone-induced modulation of nitric oxide-cGMP signaling pathway and androgenesis in the rat Leydig cells. Biology of Reproduction. 83:434–442.

Arendt J. 2005. Melatonin: characteristics, concerns, and prospects. Journal of Biological Rhythms. 20:291–303.

Baburski AZ, Sokanovic SJ, Janjic MM, Stojkov-Mimic NJ, Bjelic MM, Andric SA, Kostic TS. 2015. Melatonin replacement restores the circadian behavior in adult rat Leydig cells after pinealectomy. Molecular and Cellular Endocrinology. 413:26–35.

Baburski AZ, Sokanovic SJ, Radovic SM, Andric SA, Kostic TS. 2016. Circadian rhythm of the Leydig cells endocrine function is attenuated during aging. Experimental Gerontology. 73:5–13.

Chang HM, Huang YL, Lan CT, Wu UI, Hu ME, Youn SC. 2008. Melatonin preserves superoxide dismutase activity in hypoglossal motoneurons of adult rats following peripheral nerve injury. Journal of Pineal Research. 44:172–180.

Davidoff MS, Middendorff R, Mayer B, deVente J, Koesling D, Holstein AF. 1997. Nitric oxide/cGMP pathway components in the Leydig cells of the human testis. Cell and Tissue Research. 287:161–170.

Davidoff MS, Middendorff R, Mayer B, Holstein AF. 1995. Nitric oxide synthase (NOS-I) in Leydig cells of the human testis. Archives of Histology and Cytology. 58:17–30.

Davis FC. 1997. Melatonin: role in development. Journal of Biological Rhythms. 12:498–508.

Del Punta K, Charreau EH, Pignataro OP. 1996. Nitric oxide inhibits Leydig cell steroidogenesis. Endocrinology. 137:5337–5343.

Drewett JG, Adams-Hays RL, Ho BY, Hegge DJ. 2002. Nitric oxide potently inhibits the rate-limiting enzymatic step in steroidogenesis. Molecular and Cellular Endocrinology. 194:39–45.

Dufau ML. 1998. The luteinizing hormone receptor. Annual Review of Physiology. 60:461–496.

Esposito E, Iacono A, Muia C. 2008. Signal transduction pathways involved in protective effects of melatonin in C6 glioma cells. Journal of Pineal Research. 44:78–87.

Gambaryan S, Butt E, Marcus K, Glazova M, Palmetshofer A, Guillon G, Smolenski A. 2003. cGMP-dependent protein kinase type II regulates basal level of aldosterone production by zona glomerulosa cells without increasing expression of the steroidogenic acute regulatory protein gene. The Journal of Biological Chemistry. 278:29640–29648.

Haider SG. 2007. Leydig cell steroidogenesis: unmasking the functional importance of mitochondria. Endocrinology. 148:2581–2582.

Kostic T, Milin J, Maric D. 1997. The implication of the rat pineal gland in Leydig cells reactive response to acute immobilization. Neuroendocrinol Letters. 18:41–46.

Kostic TS, Andric SA, Stojilkovic SS. 2001. Spontaneous and receptor controlled soluble guanylyl cyclase activity in anterior pituitary cells. Molecular. Endocrinology. 15(6):1010–1022.

Kostic TS, Stojkov NJ, Janjic MM, Andric SA. 2010. Structural complexity of the testis and PKG I / StAR interaction regulate the Leydig cell adaptive response to repeated immobilization stress. International Journal of Andrology. 33:717–729.

Lu J, Cassone VM. 1993. Daily melatonin administration synchronizes circadian patterns of brain metabolism and behavior in pinealectomized house sparrows, *Passer domesticus*. Journal of Comparative Physiology A. 173:775–782.

Payne AH, Hales DB. 2004. Overview of steroidogenic enzymes in the pathway from cholesterol to active steroid hormones. Endocrine Reviews. 25:947–970.

Prendergast BJ, Pyter LM. 2009. Photoperiod history differentially impacts reproduction and immune function in adult Siberian hamsters. Journal of Biological Rhythms. 24(6):509–522.

Reiter RJ, Tan DX, Manchester LC, Qi W. 2001. Biochemical reactivity of melatonin with reactive oxygen and nitrogen species: a review of the evidence. Cell Biochemistry and Biophysics. 34:237–256.

Reiter RJ. 1991. Pineal melatonin: cell biology of its synthesis and of its physiological interactions. Endocrine Reviews. 12:151–180.

Saenz DA, Turjanski AG, Sacca GB. 2002. Physiological concentrations of melatonin inhibit the nitridergic pathway in the Syrian hamster retina. Journal of Pineal Research. 33:31–36.

Shukla P, Sun C, O'Rourke ST. 2012. Melatonin inhibits nitric oxide signaling by increasing PDE5 phosphorylation in coronary arteries. American Journal of Physiology: Heart and Circulatory Physiology. 303:H1418–H1425.

Silva CLM, Tamura EK, Macedo SM , Cecon E, Bueno-Alves L, Farsky SH, Ferreira ZS, Markus RP. 2007. Melatonin inhibits nitric oxide production by microvascular endothelial cells in vivo and in vitro. British Journal of Pharmacology. 151:195–205.

Sokanovic SJ, Baburski AZ, Janjic MM, Stojkov NJ, Bjelic MM, Lalosevic D, Andric SA, Stojilkovic SS, Kostic TS. 2013. The opposing roles of nitric oxide and cGMP in the age-associated decline in rat testicular steroidogenesis. Endocrinology. 154(10):3914–3924.

Starkey SJ, Walker MP, Beresford IJ, Hagan RM. 1995. Modulation of the rat suprachiasmatic circadian clock by melatonin in vitro. Neuroreport. 6:1947–1951.

Stumpf I, Bazwinsky I, Peschke E. 2009. Modulation of the cGMP signaling pathway by melatonin in pancreatic beta-cells. Journal of Pineal Research. 46:140–147.

Tamura EK, Silva CL, Markus RP. 2006. Melatonin inhibits endothelial nitric oxide production in vitro. Journal of Pineal Research. 41:267–274.

Tatsumi N, Fujisawa M, Kanzaki M, Okuda Y, Okada H, Arakawa S, Kamidono S. 1997. Nitric oxide production by cultured rat Leydig cells. Endocrinology. 138:994–998.

Valenti S, Cuttica CM, Fazzuoli L, Giordano G, Giusti M. 1999. Biphasic effect of nitric oxide on testosterone and cyclic GMP production by purified rat Leydig cells cultured in vitro. International Journal of Andrology. 22:336–341.

Valero M, Espine LM, Mosquera J. 2006. Melatonin decreases nitric oxide production, inducible nitric oxide synthase expression and lipid peroxidation induced by Venezuelan encephalitis equine virus in neuroblastoma cell cultures. Neurochemical Research. 31:925–932.

Weissman BA, Niu E, Ge R, Sottas CM, Holmes M, Hutson JC, Hardy MP. 2005. Paracrine modulation of androgen synthesis in rat leydig cells by nitric oxide. Journal of Andrology. 26(3):369–378.

Welsh DK, Takahashi JS, Kay SA. 2010. Suprachiasmatic nucleus: cell autonomy and network properties. Annual Review of Physiology. 72:551–577.

Yilmaz B, Kutlu S, Mogulkoç R, Canpolat S, Sandal S, Tarakçi B, Kelestimur H. 2000.Melatonin inhibits testosterone secretion by acting at hypothalamo-pituitary-gonadal axis in the rat. Neuroendocrinology Letters. 21:301–306.

Supporting information

Additional supporting information may be found in the online version of this article at the publisher's web-site:

Table S1. The primers sequences used in qPCR analysis.