Stress-induced glucocorticoids alter the rhythmic activity of Leydig cells depending on the circadian time.

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Running title: Circadian time challenges Leydig cell stress-response

Abstract

The connection between two fundamental processes in cells, the stress response and circadian timekeeping, were analyzed on Leydig cells from stressed rats. Stress increased glucocorticoids and decreased testosterone blood level. Transcriptional analysis revealed different sensitivity to stress events depending on the circadian time: the majority of steroidogenesis-related genes (*Lhcgr*, *Nr3c1*, *Cyp11a1*, *Cyp17a1*, *Hsd3b1/2*) were down-regulated by stress in the inactive (light) phase, but in the active (dark) phase of the day, they were unchanged or even up-regulated. Stress potentiates the expression of clock elements *Bmal1*/BMAL1, *Per1/2*/PER1 and *Rev-erba*. Glucocorticoid-treated rats showed a similar response, likewise stress on the negative clock regulators (*Rev-erba/b*, *Cry1/2*, *Per1/2*), suggesting that stress-induced glucocorticoids regulate clock loops but also outside genes through E-/RORE-box. Blockade of intratesticular glucocorticoid receptors prevented stress-induced change in expression negative clock regulators. The results reveal glucocorticoid-mediated communication between the stress- and circadian-system resets the Leydig cell's clock.

Introduction

Adaptation allows living beings to adjust themselves in response to persistent changes in the environment. Two systems, stress and the circadian clock are crucial for adaptation and maintenance of dynamic equilibrium continuously challenged by environmental cues. Both systems involved in synchronizing with the environment regulate body physiology differently.

Firstly, the stress system enables the organism's rapid adaptive compensatory response to demand survival in unpredictable, dangerous situations (so-called "fight and flight" response). The stress employs the fast reaction of the autonomous nervous system and adrenaline secretion followed by a delayed activation of glucocorticoids (GCs) secretion via the hypothalamic-pituitary-adrenocortical axis (Charmandari et al., 2005).

Secondly, the circadian clock enables predicting predictable environmental changes adjusted to the earth's 24-h rotation period, coordinating numerous physiologic processes, from gene expression to behavior (Agorastos et al., 2020). The complex circadian system consisting of the master clock in suprachiasmatic nucleus (SCN) and peripheral clocks in almost every cell provides a harmonious timed order of metabolic processes at different organizational levels. On the cell level, the clock system consists of clock genes coding transcription factors that operate through positive and negative transcription-translation regulatory feedback loops (Takahashi, 2017). The primary feedback loop consists of positive clock elements (BMAL1/CLOCK or NPAS2), which activate the expression of negative elements (PER1-3 and CRY1/2). Negative elements dimerize and inhibit BMAL1/CLOCK, thus shutting down their own transcription. This loop can be modified by inhibitory REV-ERB and DEC or stimulatory ROR elements (Albrecht, 2002; Ukai and Ueda, 2010). The rhythm in clock gene transcription is decoded into

physiological signals through cell-specific transcriptional activity via circadian promoter elements such as E-boxes, D-boxes, and ROREs (Ukai and Ueda, 2010). Still, the clock system works over slow expression-dependent target gene regulation similar to the glucocorticoid receptor-mediated stress response with delayed action (Koch et al., 2017).

Both systems significantly impact reproduction. In males, stress lowers serum testosterone (Andric et al., 2012; Marić et al., 1996) by action on all three reproductive axis levels (Rivier and Rivest, 1991). At Leydig cells, stress decreases steroidogenesis due to changed glucocorticoid and luteinizing receptor signaling (Andric et al., 2012). Additionally, Leydig cells steroidogenesis is a rhythmic process predominantly regulated by the rhythm of pituitary LH secretion but also by peripheral clock elements (Alvarez et al., 2008; Baburski et al., 2019). In Leydig cells, intracellular cAMP, followed by transcription of key steroidogenic proteins (*Star*, *Cyp11a1*, and *Cyp17a1*) oscillate in circadian rhythm. (Baburski et al., 2016, 2015). In serum, testosterone exerts a low amplitude oscillatory pattern (Baburski et al., 2015). Given the crucial importance of testosterone for the development and maintenance of the male phenotype, including reproductive function disturbance of circadian rhythmicity could cause the poor reproductive outcome. Modern lifestyle stress can change hormone secretions resulting in a decrease in circulating testosterone favoring the onset of infertility-related conditions. Therefore, there is a need for research linking stress and circadian disorder with testosterone production capacity. Some earlier studies suggest stress-response dependence on time of stress event (Gibbs, 1970; Zimmermann and Critchlow, 1967), pointing to the relationship between the circadian rhythm of life functions and the stress-response. However, molecular events and signaling pathways responsible for such differences are not described. Our study aimed to define the

relationship between stress response and circadian timekeeping in testosterone-producing Leydig cells.

Materials and methods

Ethical Approval

All the experiments were carried out in the Laboratory for Chronobiology and Aging, the Laboratory for Reproductive Endocrinology and Signaling, DBE, Faculty of Sciences at the University of Novi Sad. The Ethics Committee on Animal Care approved all the experimental protocols and Use at the University of Novi Sad (statement no. 01-201/3), operating under the rules of the National Council for Animal Welfare and following statements of National Law for Animal Welfare (copyright March 2009). All our experiments were performed and conducted by following 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 experiments were in adherence to the ARRIVE guidelines.

Animals

Adult, three-month-old male *Wistar* rats, bred and raised in the Faculty of Sciences' animal facility, University of Novi Sad (Novi Sad, Serbia), were used for the experiments. Rats were grown in controlled environmental conditions (22 ± 2 °C; 14h light - 10h dark cycle, lights on at 6:00 AM) and provided with food and water ad libitum. Four *in vivo* experimental approaches were applied: (1) To analyze circadian sensitivity for stress stimuli, rats were subject to immobilization stress (IMO) (Andric et al., 2012; Kvetnansky et al., 1970) for 3h in different periods during 24h (ZT0-3, ZT8-11, ZT14-17, and ZT20-23; ZT0 is a time when the light turned

on); (2) to examine the effect of IMO on rhythmic Leydig cell activity, IMO was applied from ZT0-3 followed by the analysis of rhythmic steroidogenic ability in hours after IMO session (ZT3, ZT11, ZT17, and ZT23); (3) to prove that glucocorticoids mimic IMO effects on the rhythmic function of Leydig cells, rats were treated orally with Dexason (Galenika, SRB; 0.01mg/50µL/100g BW) at ZT0 and effects were analyzed after treatment in ZT3, ZT11, ZT17, and ZT23 respectively. Control rats received 50 µL of water simultaneously as experimental rats received Dexason; (4) The contribution of glucocorticoids in the effects of stress was analyzed by local glucocorticoid receptor blockade. Rats received antagonist of glucocorticoid receptor RU486 (20 µg/20 µL DMSO/testis) into each testis, 12h before the morning IMO session, and effects were analyzed immediately after IMO. Control rats received 20 µL DMSO/testis at the same time as RU486 treated rats; (5) To obtain information about the direct effect of glucocorticoids on peripheral Leydig cell's clock and steroidogenesis, *ex vivo* treatment of primary culture with hydrocortisone wo/with RU486 (glucocorticoid receptor antagonist) were performed. The doses of dexason and hydrocortisone used in the *in vivo* and *ex vivo* experiments were selected on the basis of an approximation to the level of glucocorticoids in the blood after stress.

For *in vivo* experiments control and experimental group consisted each from 20-24 rats randomly divided into 4 time points (5-6 rats/time points). No masking was used during group allocation, data collection or data analysis. Hormone levels were measured individually (n=5-6); PCR analysis was run on samples of Leydig cells obtained form 3-4 rats, each gene was analyzed in duplicates. Previous results from our group obtained on the rat model showed that the size of the group of 5-6 rats was sufficient to calculate hormone parameters of the circadian rhythm. The

sample size was checked also by Power Analysis using G Power software (http://core.ecu.edu/psyc/wuenschk/Power.htm) according to previous results.

All *in vivo* experiments were repeated two and *ex vivo* three times.

IMO protocol

IMO was performed as described previously (Andric et al., 2012b; Kvetnansky et al., 1970). Briefly, rats were handled daily during a 3 weeks period of acclimation before experiments. After that, animals were bound in the supine position for 3h by fixing their limbs to the wooden board with thread. The head motion was not limited. Freely moving rats served as control groups in all experimental models. All activities during the dark phase were performed under the red light. After each experiment, rats were quickly decapitated without anesthesia, and the trunk blood was collected. Individual serum samples were stored at −80 °C until they were assayed for hormone levels.

Detection of an animal's voluntary activity

To analyze the voluntary rat's activity (ten days before and two days after) Dexasone application, rats were placed in individual cages with a running wheel system. The system was set to record turns of the wheel that the animal made every 6 min. Based on the collected data, the analyses were performed in Cosinor. online program (Molcan, 2019).

Preparation of purified Leydig cells

The testes were isolated, decapsulated, and the main blood vessel was removed. Decapsulated testes were placed in plastic tubes (2 testes per tube) containing collagenase solution (0.25 mg

collagenase/mL-1.5 %-BSA-20 mM HEPES-M199, Sigma, St Louise, Missouri) and incubated in a shacking-water bath (15 min/34 °C/120 cycles/min). The reaction was stopped by adding a sufficient amount of cold M199 medium, and seminiferous tubules were removed by filtration. The interstitial cells' suspension was centrifuged (160xg/5 min), and the cell pellet was resuspended in DMEM-F12 medium (Sigma, St Louise, Missouri) 8 mL/rat. The resuspended cells (35-40x10^6) were moved on the Percoll gradients (Sigma, St Louise, Missouri) and centrifuged 1100xg/28 min. After centrifugation, Leydig cells were harvested, washed with a sufficient amount of the M199-0.1% BSA, centrifuged (200xg/5 min), and resuspended in DMEM-F12 medium (5 mL/animal). In line with the procedure and according to the HSD3B staining (Payne et al., 1980) presences of Leydig cells in the culture was greater than 90%; according to the Trypan blue exclusion test (Baburski et al., 2016), viability of the cells was greater than 90%. The Leydig cell suspension was centrifuged, and the pellet was stored at -80 °C for RNA and protein analysis.

***Ex vivo* experiments**

To obtain information about the direct glucocorticoid effects on clock and steroidogenesis in Leydig cells, *ex vivo* experiments were performed using primary cultures of purified Leydig cells. Leydig cells were plated (3×10^6 cells in a 90 mm Petri dish). After a recovery period in DMEM/F12 supplemented with 10% of fetal bovine serum, the culture medium was changed with serum-free DMEM/F12 (that moment was referred to as ZT0). Leydig cells were treated with/without RU486 (1 μM; Sigma, St Louis, MO) for 15 minutes prestimulation followed by 4h stimulation with Hydrocortisone (100 nM; Sigma, St Louis, MO). Experiments were performed

at ZT4, ZT14, and ZT24. After the stimulation period, cells were collected in indicated time points and used for gene expression analysis. *Ex vivo* experiments were repeated for two times.

RNA isolation, cDNA preparation, and gene expression analyzing

Total RNA was isolated by using Trizol (TermoFisher Scientific, Waltham, USA) or Rneasy Mini Kit (Quiagen, Germantown, USA) according to manuals instructions. The total RNA's amount and purity were assesed by spectrophotometric measurement (NanoDrop, Shimadzu), and DNase I treatment was performed according to the Invitrogen manufacturer's protocol (Invitrogen, Carlsbad, USA). cDNA was synthesized using the Applied Biosystems kit (Termo Fisher Scientific, Massachusetts, USA) according to the manufacturer's instructions. Relative gene expression was determined by RQ-PCR using SYBR-Green based technology (Applied Biosystems/Termo Fisher Scientific, Massachusetts, USA). With all the necessary compounds, PCR reaction was performed in presence of 5 µl/25 ng cDNA and 500 nM specific primers. The relative quantification of each gene was performed in duplicate and *Gapdh* was used as an endogenous control. The primer sequences and gene bank accession code for full gene sequences were downloaded from www.ncbi.nlm.nih.gov/sites/entrez, and they are presented in Suppl.Table1.

Protein extraction and western blot analysis

Platted cells were lysed by buffer (20 mM HEPES, 10 mM EDTA, 2.5 mM $MgCl_2$, 1 mM DTT, 40 mM β-glicerophosphate, 1% NP-40, 2 µM leupeptin, 1 µM aprotinin, 0.5 mM AEBSF, phosphatase inhibitor cocktail (PhosSTOP, Roche, Basel, Switzerland) and the lysates were uniformed by Bradford method (Andric et al., 2007; Kostic et al., 2010). Samples were mixed

with SDS protein gel loading solution (Quality Biological, Gaithersburg, USA), boiled for 3 min, and separated by one-dimensional SDS-PAGE electrophoresis (Bio-rad, Berkeley, USA). Transfer of the proteins to the PVDF membrane was performed by electroblotting overnight at 4°C/40A, and the efficacy of transfer was checked by staining and distaining the gels. The membranes were blocked by 3% BSA-1xTBS for 2h/room temperature, incubation with primary antibody was done overnight at 4 °C, and 0.1% Tween-1xTBS was used for washing membranes incubation with secondary antibody was done for 1h/room temperature (Andric et al., 2012). Immunoreactive bands were detected using luminol reaction and MyECL imager (www.Fischer.sci)/films and densitometric measurements were performed by Image J program version 1.32 (https://rsbweb.nich.gov//ij/download.html). Anti-BMAL1 purchased from Abcam, (Cambridge, UK, #ab49421, dilution 1:800); anti-PER1 from Abcam, (Cambridge, UK, #ab3443, dilution 1:500); anti-ACT purchased from Santa Cruz Biotechnology (Heidelberg, Germany, #sc-8432)

Hormone and glucose measurements

Serum testosterone levels were measured by radioimmunoassay. Testosterone levels are referred to as testosterone + dihydrotestosterone (T + DHT), because the anti-testosterone serum № 250 showed 100% cross-reactivity with DHT (Kostic et al., 2010). All samples were measured in duplicate in one assay (sensitivity: 6 pg per tube; intraassay coefficient of variation 5–8%). For serum corticosterone levels (Stojkov et al., 2012), all samples were measured in duplicate, in one assay by the corticosterone EIA Kit (Caymanchem, Michigan, USA) with 30 pg/ml as the lowest standard significantly different from blank. The level of glucose in serum was estimated by using

BAYER Contour plus Glucose Monitoring System (Bayer, Basel, Switzerland; measuring range 0.6-33.3 mmol/L). Technically, all samples obtained individually, were measured in duplicate.

Statistical and rhythm analysis

The results represent group means ± SEM values of three in vivo experiments. Rhythm parameters (p, MESOR, amplitude, and acrophase) were obtained by cosinor.lm() function from the "cosinor" package (Sachs, 2015) in the R environment and by using online Cosinor.Online software fitted to 24 h period (http://www.circadian.org/softwar.html) (Molcan, 2019) Statistical significance ($p < 0.05$) between the treated group and control group within the same time point was analyzed using Mann–Whitney test.

Principal component analysis (PCA) was done with dudi.PCA() function implemented in "ade4" package (Dray and Dufour, 2007), on scaled and centered data matrix, within the R environment. We decided to retain the first two PC based on eigen values and cumulative variation. In support of such a decision, we performed Horn's parallel analysis for a PCA with the "paran" package, to adjust for finite sample bias in retaining components (Dinno, 2018). Biplots visualization were done with "factoextra" package (Kassambara and Mundt, 2020).

Results

Sensitivity to stress depends on the circadian time

To examine whether the circadian time of stress event can provoke different stress-responses, rats were immobilized in different periods during 24h, and responses analyzed immediately after IMO session. In the serum of control rats, circadian fluctuations of corticosterone, glucose, and testosterone levels were observed (Fig. 1A&B&C; the rhythm parameters in Suppl. Table 2) with

a peak around ZT11 for all three. IMO treatment effectively increased corticosterone and glucose levels in all investigated ZT, although glucose levels were more pronounced in the ZT3 and ZT23. On the contrary, IMO reduced testosterone levels in serum in all investigated ZTs (Fig. 1C).

The relative ratio between testosterone and corticosterone, calculated on ZT3 value in control samples ($T/C_{ZT3} = 1$) showing circadian variation of T/C, which depicts circadian fluctuations in blood testosterone and corticosterone. The highest T/C observed was at ZT11 ($T/C_{ZT11} = 1.6$). However, T/C was reduced by IMO approximately 38-fold, depending on the time IMO event. The lowest T/C value was observed in ZT3 ($T/C_{ZT3} = 0.02$), while the highest was observed in ZT11 ($T/C_{ZT11} = 0.03$).

To detect a possible different stress effect on the gene expression essential for the Leydig cells' endocrine function, we perform transcriptional analysis in Leydig cells and pituitary originating from control and IMO rats.

Results showed pronounced circadian rhythm in the transcription of pituitary genes encoding LH subunits (*Cga, Lhb*), the principal regulator of Leydig cell function (Fig.1 D, E; please see rhythm parameters in Suppl.Table2). IMO significantly decreased *Cga* at ZT11 and ZT17 (Fig. 1D) and *Lhb* at ZT17 and ZT23 (Fig. 1E). Additionally, transcription of genes encoded receptors important for Leydig cell endocrine activity was monitored. The *Lhcgr*, which determines Leydig cells' sensitivity on LH, did not show circadian oscillation (Fig. 1F), while *Nr3c1* enabling reactivity to corticosterone had 24h oscillatory pattern (Fig. 1G). IMO significantly decreased the transcription of genes for both receptors in the light phase, while the dark phase was without effect (Fig. 1 F, G).

The transcription of critical steroidogenic genes, *Star*, *Cyp11a1*, *Cyp17a1* showed 24-oscillations with a peak approximately in the middle of the light phase (Fig. 1 I, J, L; Suppl.Table2) i.e., a few hours before the peak of testosterone in serum. IMO changed the pattern of rhythmic expression of the *Star*, *Cyp11a1*, and *Cyp17a1* underlying a pronounced reduction in the light phase (Fig. 1 I, J, L). However, results did not support the circadian oscillatory pattern in the transcription of *Hsd3b1/2* IMO significantly reduced expression during light phase (Fig 1. K).

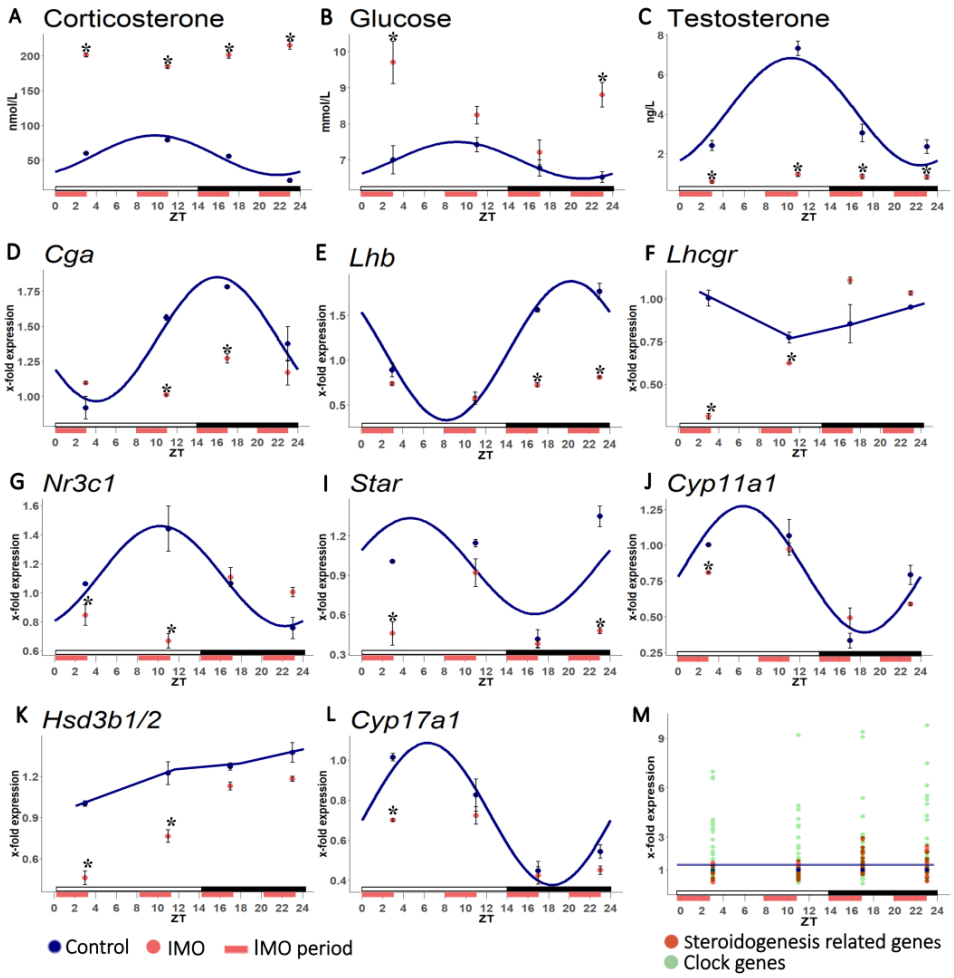

Fig 1. Effect of IMO on steroidogenesis-related genes depends on circadian time.
Wistar rats were subject to IMO for three hours in four time points during the 24h (ZT0-3, ZT8-11, ZT14-17, ZT20-24) and serum was collected for determination of corticosterone (A), glucose (B), and testosterone (C) levels. Pituitary and Leydig cells were collected, RNA isolated followed by RT and RQ-PCR analysis of steroidogenesis-related genes expression (D, E, F, G, H, I, J, K, L). Data points are group mean ± SEM values (n= 5 for A, B, C and n = 3 for D, E, F,

G, H, I, J, K). In this and Figure 2, the best fitting curves are composed by Cosinor package in R studio. For rhythm parameters (Robustness, MESOR, Amplitude, and Acrophase) please see Suppl.Table2. *Statistical significance between control and IMO groups for the same time point ($p < 0.05$). ZT in this and all other figures is Zeitgeber time. The data for this and following figures can be seen in source data files as well as in the UNS repository (https://open.uns.ac.rs/).

To estimate the effect of IMO on the peripheral clock in Leydig cells, transcription analysis of canonical clock genes was done (Fig. 2; Suppl.Table2).

The majority of examined genes (*Bmal1*, *Per1/2*, *Cry1/2*, *Rev-erba/b*, *Rob*) had a rhythmic expression in rat Leydig cells (Fig. 2 A, D, E, F, G, H, I, K; Suppl.Table2). On the contrary, *Clock*, *Npas*, and *Rora* transcripts did not follow any significant pattern (Fig. 2 B, C, J; Supplement Table 2). *Bmal1* reaches peak at the beginning while *Per1/2, Cry2, Rev-erba/b, and Rorb* at the end of the light phase, followed by *Cry1* rise in the dark phase. Generally, IMO changed transcription of clock genes in Leydig cells: *Bmal1* was increased (Fig. 1A), and *Clock* (Fig. 1B) decreased immediately after morning IMO (ZT3) while its counterpart *Npas2* was unaffected by IMO situations (Fig. 2C). However, clock negative regulators were enhanced: *Per1* after all IMO sessions regardless of the time of occurrence (Fig. 2D), and *Per2, Cry1, and Cry2* at ZT3 and ZT23 (Fig. 2E, F, G). Additionally, IMO affected transcription of genes in the circadian clock's secondary loop by increased expression of *Rev-erba* at all tested times (Fig. 2H) and *Rorb* at ZT17 (Fig. 2K). Changed protein levels of BMAL1 and PER1 due to IMO were confirmed by western blot analysis. As expected, BMAL1 and PER1 showed circadian profiles in the control samples with an increased level of BMAL1 in the light in relation to the dark phase and vice versa situation for its negative regulator PER1 (Fig. 2L, M). IMO increased BMAL1 in the dark period of the day (Fig. 2L) while PER1 was increased in all examined time points (Fig. 2M).

In summary, the results suggest different sensitivity to IMO depending on the circadian time of the event. Transcriptions of most steroidogenesis-related genes were down-regulated by IMO in the light but unchanged or even up-regulated in the dark phase of the day (Fig. 1M).

Results suggested that IMO potentiates expression of clock elements that act over E-box (BMAL1 and PER1) and transcription of *Rev-erba*, which exerts its action via RORE-box, suggesting a possible effect on gene expression not only on the clock system but also outside it.

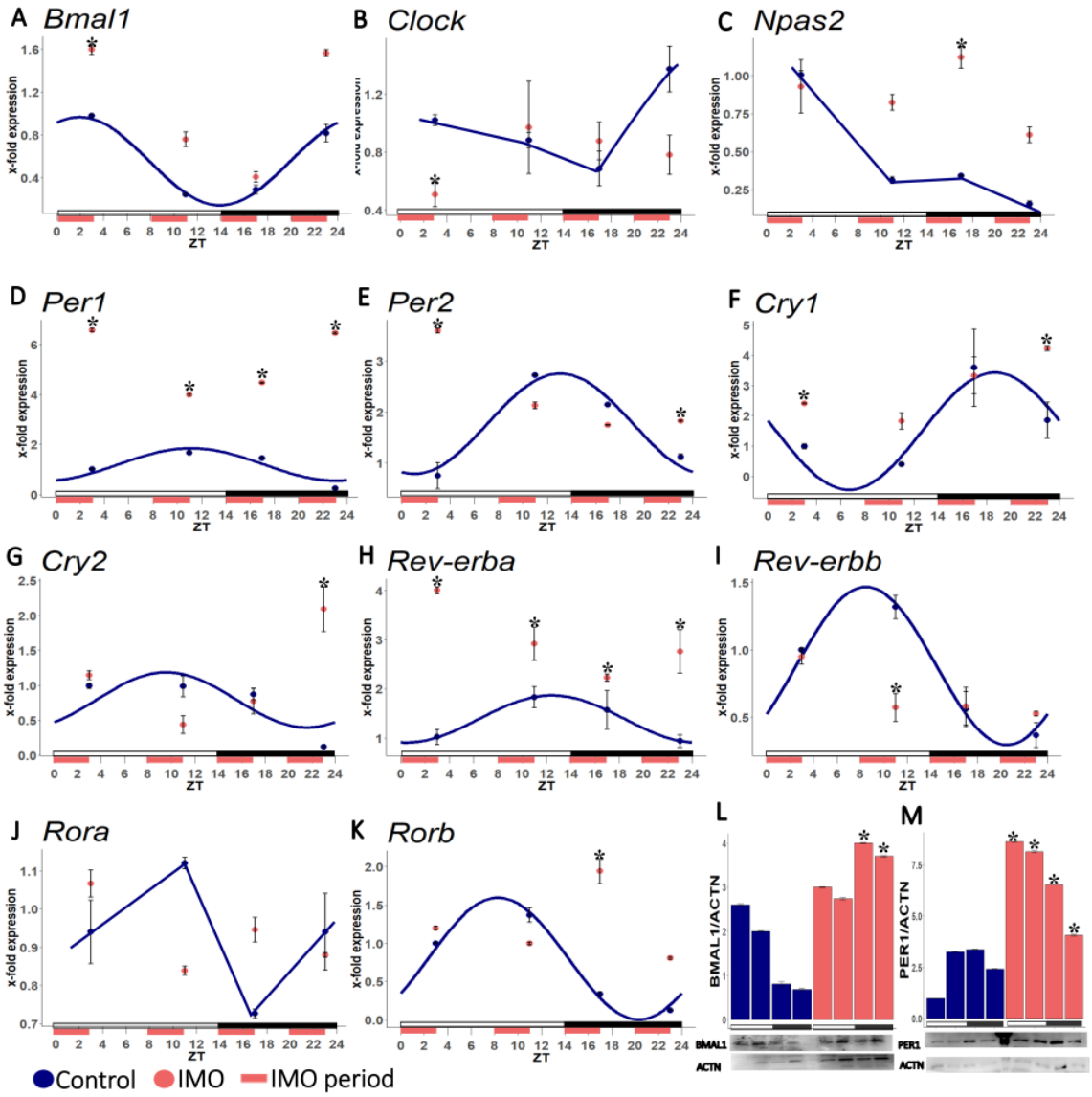

Fig 2. Effect of IMO at different circadian times on transcription of clock genes. *Wistar* rats were subject to IMO for three hours in four time points during the 24h (ZT0-3, ZT8-11, ZT14-17, ZT20-24). Leydig cells were purified, RNA extracted and used for RT followed by RQ-PCR analysis of clock genes expression (D, E, F, G, I, J, K). Data points are group mean ± SEM values, (n = 3). For rhythm please see Suppl. Table 2. *Statistical significance between control and IMO groups for the same time point ($p < 0.05$). The protein level of BMAL1 (L) and PER1 (M) were determined by western blot. Representative blots are shown. Bars above blots represent mean ± SEM values from pooled data using scanning densitometry values normalized on ACTB. * Statistical significance between control and IMO group at the same time point ($p < 0.05$).

Effect of morning IMO on Leydig cell's circadian rhythm

Since IMO during the light phase reduced transcription of the majority of steroidogenesis-related genes, we aimed to determine the consequences of the Leydig cells' rhythmic activity. In that respect, rats were subject to IMO from ZT0-3, and transcriptional pattern of genes important for rhythmic Leydig cell's endocrine activity together with hormones blood level was monitored within 24 h after IMO.

Morning IMO changed oscillatory profile of corticosterone, glucose, and testosterone blood levels (Suppl.Table 3): mesor and amplitude of corticosterone blood level were increased and the acrophase shifted to the left, ie towards the end of the IMO (Fig. 3A); changes in glucose profile were similar to corticosterone (Fig. 3B) while the amplitude and mesor of testosterone secretion decreased (Fig. 3C). The T/C values in the first 20h after IMO showed disturbrd oscillatory pattern, with decreased values at the ZT3, ZT17 and ZT23 but increased at ZT11 in respect to control values (after morning IMO: $T/C_{ZT3} = 0.11$, $T/C_{ZT11} = 2.23$, $T/C_{ZT17} = 0.21$, $T/C_{ZT23} = 0.30$ vs. Control: $T/C_{ZT3} = 1$, $T/C_{ZT11} = 1.5$, $T/C_{ZT17} = 0.89$, $T/C_{ZT23} = 0.83$, respectively).

The rhythmic profile of pituitary *Cga* and *Lhb* also changed by morning IMO (Suppl.Table3): the acrophase of *Cga* transcription shifted to the beginning of the dark period (Fig. 3D), while the *Lhb* completely changed phase and reduced mesor and amplitude (Fig. 3E). Those changes

are suggesting a possible decrease in LH synthesis. Leydig cell's *Lhcgr* decreased (Fig. 3F) while *Nr3c1* dropped and lost rhythmic transcription characteristics (Fig. 3G, Suppl.Table3). Reduced amplitude and mesor of *Star* (Fig. 3H), *Cyp11a1* (Fig. 3I), and *Cyp17a1* (Fig. 3K) were noticed. IMO in the morning decreased arrhythmic transcription of *Hsd3b1/2* (Fig. 3J).

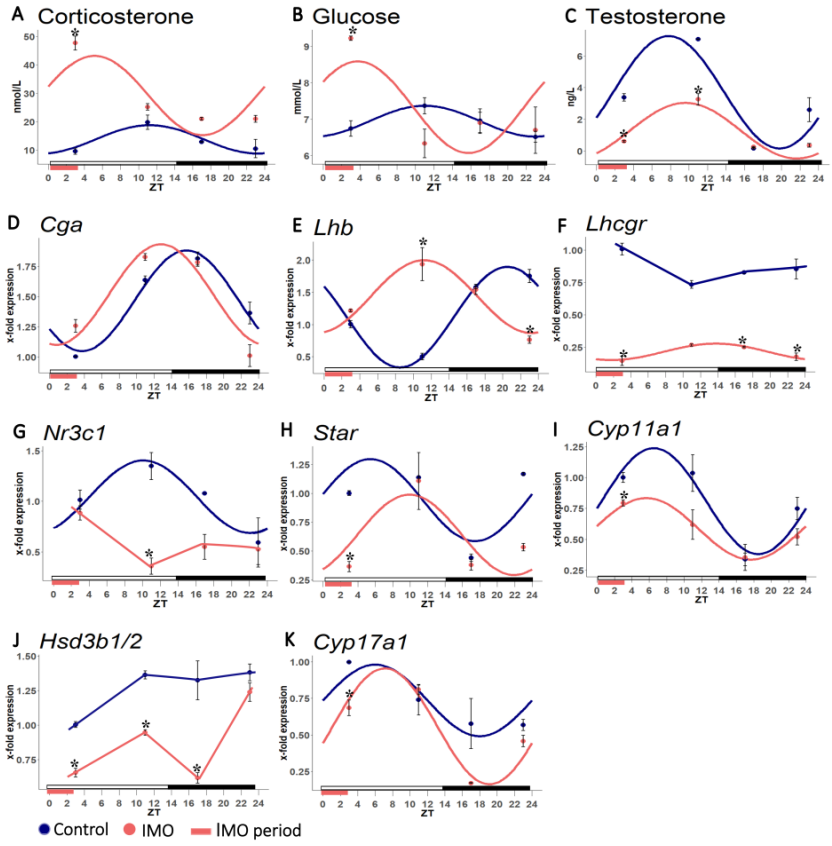

Fig 3. Morning IMO changed transcription rhythm of steroidogenic-related genes. IMO was applied from ZT0-ZT3 and animals were scarified in four time points during the 24 h (ZT3, ZT11, ZT17, ZT24) and serum was collected for determination of corticosterone (A), glucose (B) and testosterone (C) levels. Pituitary and Leydig cells were collected, RNA isolated for RT followed by RQ-PCR analysis of steroidogenesis-related genes expression (D, E, F, G, H, I, J, K). Data points are group mean ± SEM values (n=5 for A, B, C and n = 3 for D, E, F, G, H, I, J, K). In this and figure 4, the best fitting curves are composed by Cosinor method. For rhythm parameters please see Suppl. Table 3. *Statistical significance between control and IMO groups for the same time point ($p < 0.05$).

At the beginning of the light period, IMO remarkably affected transcription Leydig cell's clock genes (Suppl.Table3). Increased *Bmal1*, *Per1/2*, and *Rev-erba* significantly influenced rhythm characteristics by moving the acrophase towards the IMO's end (Fig. 4A, D, E, H, respectively). Decreased *Clock* detected after IMO, recovered and returned to control values (Fig. 4B); *Cry1* and *Cry2* lost rhythmic transcription (Fig. 4F, G) while *Rev-erbb* was reduced in the hours after IMO (Fig. 4I) and *Rora* and *Rorb* transcription were arrhythmic (Fig. 4J, K). In the first 20h following IMO, PER1 protein level was increased (Fig. 4L).

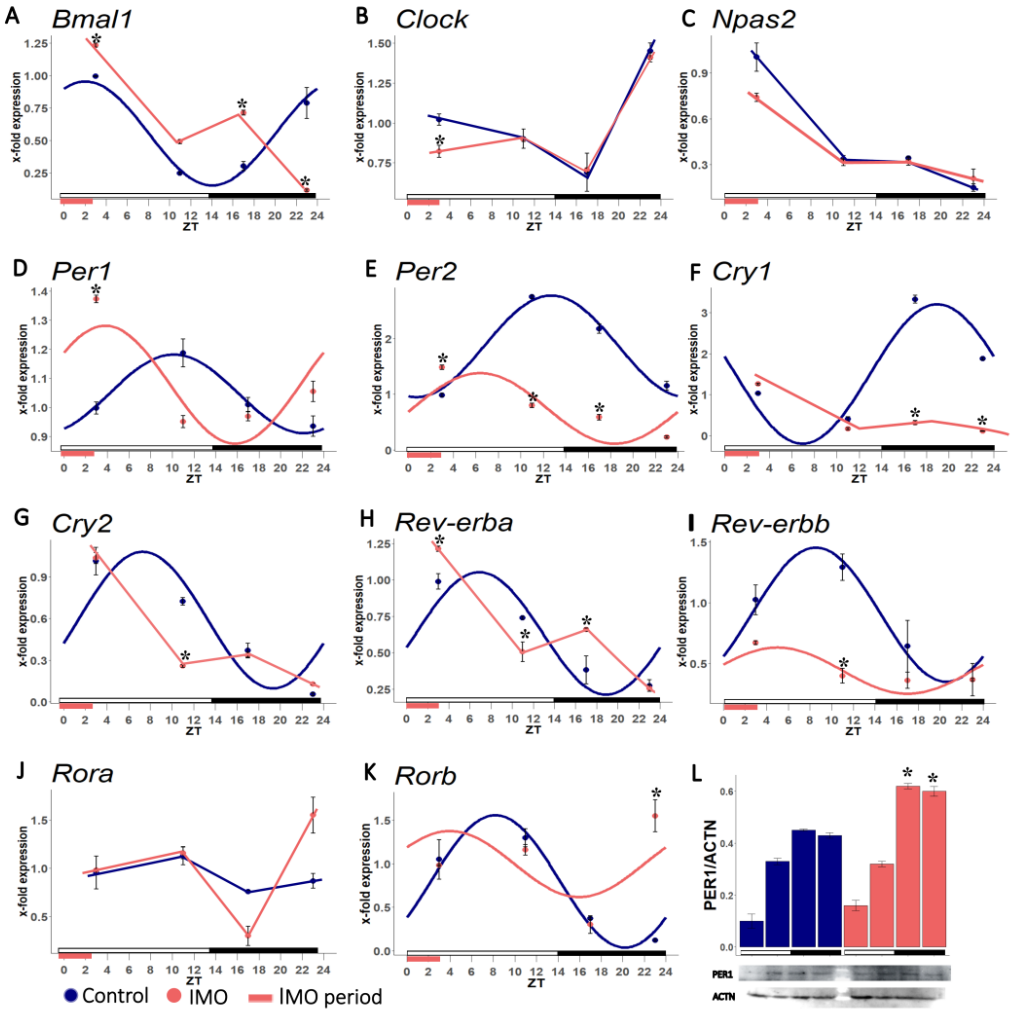

Fig 4. Morning IMO changed transcription rhythm of clock genes. IMO was applied from ZT0-ZT3 and animals were sacrificed in four time points during the 24 h (ZT3, ZT11, ZT17, ZT24). Leydig cells were purified, RNA extracted, used for RT and consequently RQ-PCR analysis of clock genes expression (A, B, C, D, E, F, G, H, I, J, K). Data points are group mean ± SEM values (n = 3). For rhythm parameters please see Suppl.Table 3. The protein level of PER1 (L) was determined by western blot. Representative blots are shown. Bars above blots represent mean ± SEM values from pooled data using scanning densitometry values normalized on ACTB. *Statistical significance between Control and IMO group at the same time point ($p < 0.05$).

Effect of glucocorticoids on Leydig cell's circadian rhythm

It is well established that glucocorticoids, together with adrenaline, are the primary hormonal mediators of stress-reactive Leydig cells response (Andric et al., 2012). To analyze the similarity

of the glucocorticoid and IMO action on testosterone production and clock in Leydig cells, rats were treated with dexason at ZT0, and effects analyzed after treatment in ZT3, ZT11, ZT17, and ZT23, respectively.

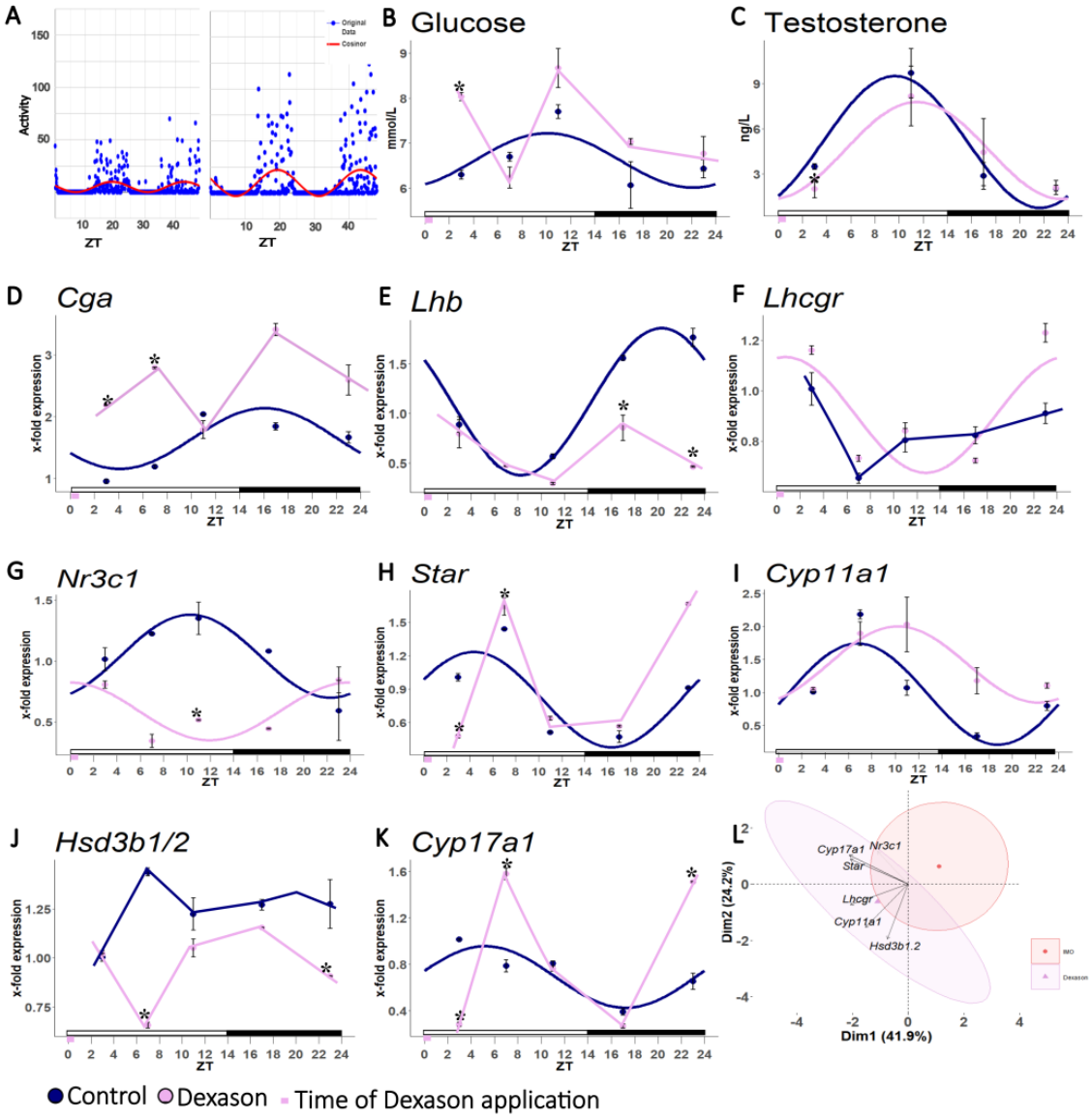

Fig 5. Treatment with glucocorticoids changed transcription rhythm of steroidogenic-related genes. *Wistar* rats were treated *per os* Dexason (0.01mg/50µL/100g BW) at ZT0 and effects were analyzed after treatment in (ZT3, ZT11, ZT117, ZT24). The voluntary rat's activity 48h before and 48h after Dexason application was recorded; the representative individual graf is shown (A) while rythm parameters are shown in Suppl. Table 4. Serum was collected for determination of glucose (B) and testosterone (C) levels. Pituitary and Leydig cells were purified, RNA isolated and used for RT followed by RQ-PCR analysis of genes expression. Data points are group mean ± SEM (n=5 for B, C and n = 3 for D, E, F, G, I, J, K). *Statistical

significance between control and Dexason groups for the same time point ($p < 0.05$). ZT in this and all other figures is Zeitgeber time. In panel K, Dim1 and Dim2 represents the first two PC and % of retained variation. cos2 estimates the qualitative representation of variables. For rhythm parameters please see Suppl. Table 5 and 6. PCA of steroidogenic-related genes in Leydig cells from IMO- and Dexason-treated groups (K); Dim1 and Dim2 represents the first two PC and % of retained variation. cos2 estimates the qualitative representation of variables. *Statistical significance between control and Dexason groups for the same time point ($p < 0.05$).

The efficacy of treatment was verified by the voluntary rat's activity 48h before and 48h after Dexason application (Fig. 5A) and high and arrhythmic blood glucose profile (Fig. 5B). Dexason treatment increased rat's activity about 2.5 times comparing to control (875.6±239.1 vs 2142.3±573.9) (Fig. 5A: Suppl.Table4) and disturbed testosterone's circadian rhythm in blood by decreasing mesor and amplitude, followed by delayed acrophase (Fig. 5C; Suppl.Table4). Pituitary *Cga* (Fig. 5D), *Lhb* (Fig. 5E) as well as Leydig cells *Lhcgr* (Fig. 5F) lost circadian transcriptional pattern, while the profile of *Nr3c1* was reduced and reversed (Fig. 5G). Circadian profile of *Star* (Fig. 5H) and *Cyp17a1* (Fig. 5K) was abandoned while *Cyp11a1* was changed with decreased mesor and delayed acrophase (Fig. 5I) by glucocorticoids treatment. Additionally, the arrhythmic expression of *Hsd3b1/2* decreased compared with control values (Fig. 5J). Finally, a comparison of the IMO and glucocorticoid effects on steroidogenesis-related gene transcription by PCA (Fig. 5L) shows that IMO and dexason clusters tend to separate across PC1. Even though clusters are not entirely distinct, a shift across PC1 is evident. The main drivers of such change are the *Lhcgr*, *Star*, and *Cyp17a1* genes relative expression. The *Nr3c1*, *Cyp11a1*, and *Hsd3b1/2* seem to be less distinct between treatments (Suppl.Table 5). Moreover, dexason changed cyclic expression of clock genes: *Bmal1*, *Clock*, *Npas2*, *Per1*, *Cry2*, *Rev-erb*, and *Rob* increased at ZT3; *Cry1* and *Rev-erba* lost the rhythmic pattern (Suppl.Table4; Fig. 6).

Comparison of the IMO and glucocorticoid effects on clock gene by PCA showed no substantial differences between experimental groups (Fig. 6L). The first two PC accounted for 72.66% of the total dataset variability. Interestingly, *Per1* is characterized by the mildest contribution to achieved variability in the first two components. Variable loadings are shown in Suppl.Table 6.

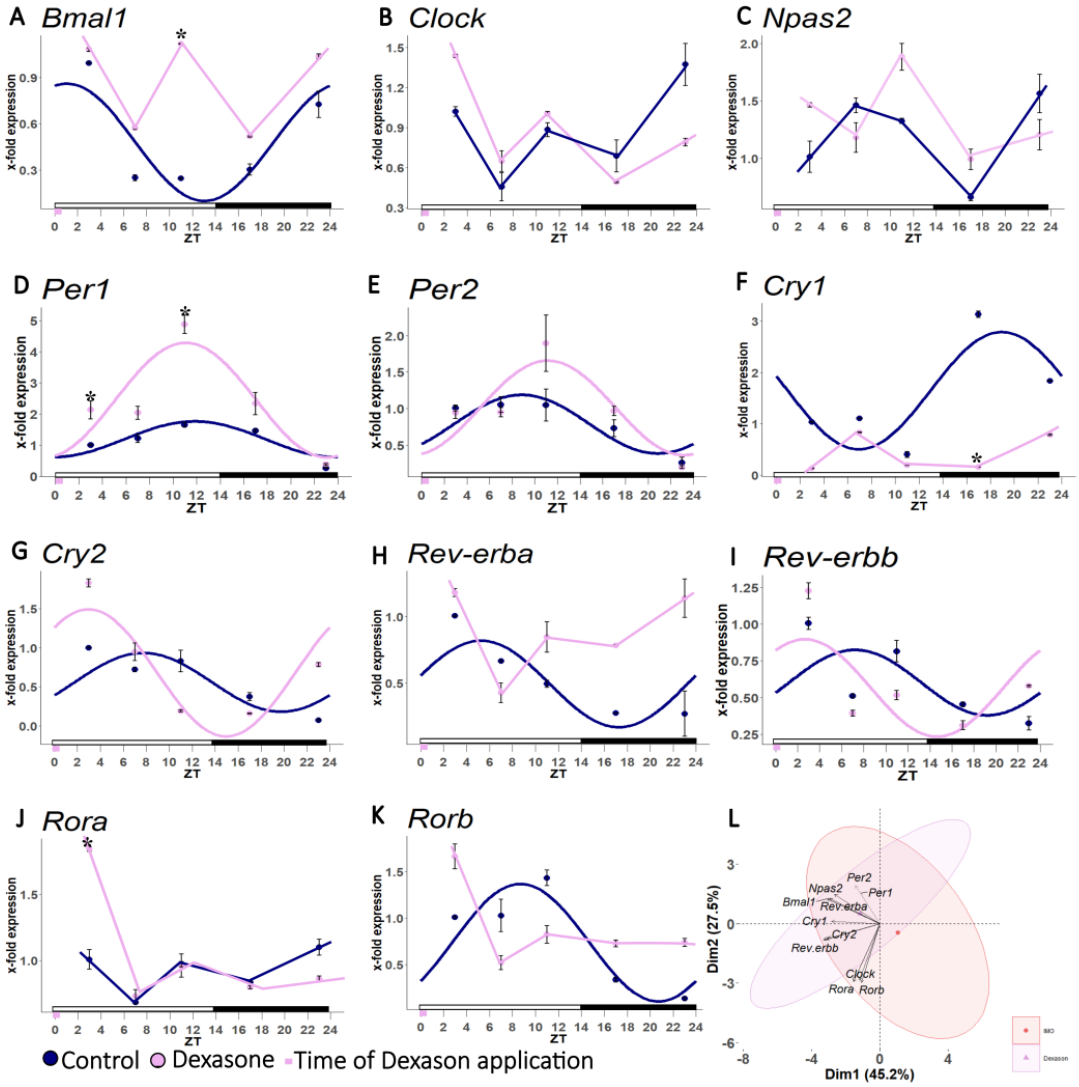

Fig 6. Treatment with glucocorticoids changed transcription rhythm of clock genes. *Wistar* rats were treated *per os* Dexason (0.01mg/50µL/100g BW) at ZT0 and effects were analyzed after treatment in ZT3, ZT11, ZT117, and ZT24. Leydig cells were purified, RNA isolated and used for RT followed by RQ-PCR analysis of clock genes expression (A, B, C, D, E, F, G, I, J). For rhythm parameters please see Suppl. Table 4. Data points are group mean ± SEM values (n = 3). PCA of clock genes in Leydig cells from IMO- and Dexason-treated rats (L); Dim1 and Dim2 represents the first two PC and % of retained variation. cos2 estimates the

qualitative representation of variables. *Statistical significance between control and IMO groups for the same time point ($p < 0.05$).

Role of testicular GRs in stress-induced changes of Leydig cell's clock.

To estimate glucocorticoids' possible contribution in IMO-induced changes in Leydig cell clock, the intratesticular treatment with RU486, the potent blocker of classic GRs (Andric et al., 2012) was performed 12 h before IMO. Such a treatment prevented IMO-induced changes in expression of *Per1/2*, *Cry1/2*, *Rev-erba/b* (Fig. 7A) in the Leydig cells, confirming that this effect is related to GR activation.

Further, to estimate the direct effect of glucocorticoids on Leydig cell's clock, Leydig cells' primary culture was incubated with hydrocortisone wo/with RU486. As expected, hydrocortisone increased transcription of *Per1*, *Per2*, and *Rev-erba*, but RU486 attenuated the hydrocortisone-induced increase in mRNA transcript for *Per1*, *Per2 Rev-erba* (Fig. 7B), validating the direct effect of GR-signaling on Leydig cell's clock.

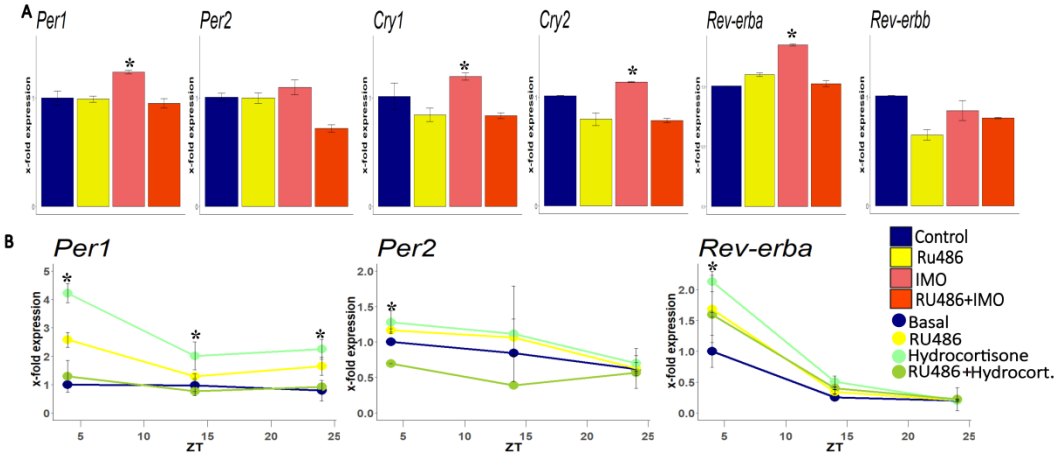

Fig.7. Direct effects of IMO-induced glucocorticoids on Leydig cell GR. RU486 (20 µg in 20µl/testis) or vehicle (20 µl/testis) was injected intratesticularly 12 h before the IMO session; at the end of the IMO session Leydig cells were prepared, RNA isolated for RT followed by RQ-PCR for monitoring of clock gene expression (A). Data bars represents means are group mean ± SEM values (n = 3). Leydig cells were isolated from adult rat testes and plated ($3x10^6$ Leydig cells/well). After 6 h of resting period in 10%FBS-DMEM/F12, cell media was changed

with fresh DMEM/F12 and that time point was referred to ZT0. Leydig cells were stimulated w/wo hydrocortisone (100 nM) or in combination w/wo RU486 (1 μM) at ZT0, ZT10, ZT20. After 4h of stimulation Leydig cells were washed and stored at -80C for RNA isolation. RQ-PCR was performed in presence of primers specific for *Per1*, *Per2*, *Rev-erba* (B). Data points are group mean ± SEM values of three independent experiments. *Statistical significance between control and experimental groups ($p < 0.05$).

Discussion

Circadian organization of the reproductive axis is essential for reproductive function (Sen and Hoffmann, 2020). Data indicates that core clock genes, *Bmal1*, *Rev-erba*, strongly influence male reproductive competency (Alvarez et al., 2008; Hodžić et al., 2013; Liang et al., 2013), especially testosterone production. However, the integrated network of signals involved in entrainment peripheral oscillators, especially in testosterone-producing Leydig cells, is presently not well understood. Here we analyzed the effect of stress on circadian oscillator in Leydig cells.

Results from the present study strongly suggest circadian time as the critical element in determining the stress response. Namely, in Leydig cells, genes responsible for testosterone production (*Star*, *Cyp11a1*, and *Cyp17a1*) together with canonical clock genes (*Bmal1*, *Per1/2/3*, *Cry1/2*, *Rev-erba/b*, *Rorb*, *Dec1/2*, *Dbp*, and *E4bp4*) are expressed in a circadian manner (Baburski et al., 2016, 2015). However, these genes' expression pattern is changed due to stress and varies depending on the time of the stressful event. In the inactive (light) phase, the key steroidogenesis-related genes (*Lhcgr*, *Cyp11a1*, *Cyp17a1*, *Hsd3b1/2*) were down-regulated, but in the active (dark) phase of the day, they were unchanged or even up-regulated. It is challenging to interpret obtained results since gene expression is a multi-regulated process involving several signaling pathways directed by hormones, especially LH, PRL corticosterone, and many others, all of which vary in circadian rhythm and could be affected by IMO. Besides, clock system

interacts with many different signals to produce an integrated output over the 24h cycle, entraining other cyclic activities in the cell (Ruginsk et al., 2018).

It is well known that all steroidogenic events in Leydig cells are initiated and depend on LH-receptor activation, including expression of proteins involved in steroidogenesis such are *Star*/StAR, *Cyp11a1*/CYP11A, *Hsd3b*/HSD3B, *Cyp17a*/CYP17A, and *Hsd17b*/HSD17B (Payne and Hales, 2004). However, obtained results indicate severe inhibitory IMO effects on *Lhcgr* transcription in inactive (light) but not in the active period. After morning IMO, expression of *Lhcgr* stayed reduced for the whole 24h period. Since LH-receptor signaling is crucial for steroidogenic genes transcription and steroidogenesis activation (Dufau, 1998; Zirkin and Papadopoulos, 2018) *Lhcgr* decreased expression in the inactive period could make a difference in respect to IMO in the active period. Also, IMO decreases transcription of pituitary *Cga* during the inactive phase and, *Lhb* has been reduced by evening IMO both likely to have consequences for the quantity of pulsatile LH secretion and impaired Leydig cells stimulation.

Nevertheless, the IMO is changing the circadian organization of clocks in the Leydig cells. IMO potentiates the expression of *Bmal1*/BMAL1 and *Per1/2*/PER1 and *Rev-erba*, suggesting that stressful stimuli can entrain the clock in the Leydig cells. Further, BMAL1 is cyclically expressed in the rat (Baburski et al., 2019, 2015) and mouse (Chen et al., 2017) Leydig cells, stimulating StAR expression (Alvarez et al., 2008), therefore contributing to the cyclic expression of StAR protein. Since IMO in the dark phase promotes BMAL1, this may mitigate the effects of stress on steroidogenesis during the night. Additionally, the presence of clock-controlled E-box, and RORE motifs, in the promoters of *Star*, *Cyp11a1*, and *Cyp17a1* (Chen et al., 2017) but also in promoters of steroidogenic regulators such as *Nur77* indicates that the BMAL1, PERs, and REV-ERBs in Leydig cell may regulate the expression of these genes and

possible changes in their intracellular level may affect the rhythm of steroidogenesis. Indeed, further studies are necessary to confirm this hypothesis.

Most of the IMO effects on Leydig cells are exerted through increased corticosterone secretion. The corticosterone inhibits steroidogenic enzymes' activity and transcription and initiating Leydig cells apoptosis. (Andric et al., 2012; Ge et al., 2005). Among being part of the stress system, corticosterone synthesis is regulated by the circadian system (Dickmeis, 2009). It is secreted in a diurnal pattern, with peak levels linked to the activity phase's start. In turn, the rhythmically released corticosterone might contribute to synchronizing the cell-autonomous clocks in the body, spreading the time information to target cells (Dickmeis et al., 2013). Further, circadian rhythmicity of *Nr3c1* transcription in Leydig cells show a very similar pattern as the corticosterone in blood, providing a diurnal effect on Leydig cells. The circadian fluctuation of testosterone shows low amplitude also peaked at the beginning of the active phase. Although important for psychiatric disease identification, the T/C ratio may indicate steroidogenic pressure to which target cells, especially cells of the reproductive system, are exposed. The T/C rate in rat circulation is highest at the beginning of the active period of the day. IMO reduced T/C with the lowest value at the beginning of the rest phase overlapping with Leydig cells' steroidogenic genes inhibition.

At the transcriptional level, glucocorticoids synchronize peripheral oscillators, influencing clock genes' expression in response to a series of conditions (Balsalobre et al., 2000). In Leydig cells, glucocorticoids released in a stress response increased and abolished the rhythmic transcription of *Bmal1*, *Cry1/2*, and *Rev-erba*, and increased *Per1*, indicating that could serve as a resetting cue. Moreover, the cosinor-rhythmometry revealed phase advance of *Per1*, after IMO or glucocorticoids treatment. The activation of GR mediates this action. It is confirmed by the *in*

vivo blockade of the testicular GR during IMO and *ex vivo* treatment of the primary Leydig cell culture with corticosterone and GR-blocker. Also, morning *in vivo* treatment with glucocorticoids evoked a similar response as IMO on the clock genes. The PC analysis displayed an absence of significant differences between IMO and glucocorticoid treatment on Leydig cell clock gene expression (Fig. 6L). Similar changes in the transcription of clock genes suggest that IMO exerts an effect on the clock gene by increasing blood glucocorticoids. Obtained results are in accordance with findings in peripheral (Chrousos, 2011; Kino, 2012). It was shown that the GR affects the expressions of *Per* genes (So et al., 2009; Yamamoto et al., 2005). Besides, the GR also regulates the expression of *Rev-erbα* (Oster et al., 2017; Torra et al., 2000).

Results indicate that glucocorticoids and GR are important in shaping clock rhythm, but they are not the only elements involved in Leydig cells' rhythm modulation. The PC analysis showed similar IMO and glucocorticoid treatment effects on *Nr3c1*, *Cyp11a1*, and *Hsd3b1/2* expression in Leydig cells. However, the results differ in part in *Lhcgr*, *Star*, and *Cyp17a1* genes relative expression, indicating other stress-altered hormones' involvement.

Anyway, glucocorticoids shape the circadian rhythm of many body functions, including willing activity based on cosinor rhythmometry. Treatment with glucocorticoid agonist increased volitional rat activity during 48h recording, confirming a long-lasting effect presumably mediated by GR's genomic effect.

The exposure to increased glucocorticoid levels, either induced by stress or exogenous administration, significantly interfere with the clock in Leydig cells and testosterone production and, therefore, with the reproductive process, with a likely impact on fertility (Sciarra et al., 2020). A timed hormone release within the reproductive axis is required for normal fertility (Sen and Hoffmann, 2020). The stress causes phase shifts or the loss of oscillations in the

transcription of clock genes when it happened at the beginning of the inactive period but had a mild effect on Leydig cells` peripheral clock at the start of the active period. Also, steroidogenic genes are much more affected by stress in the rest than in the active phase pointed the relevance of the stress event's timing. The obtained results pointed to stress and the circadian system's coordinated action in shaping male reproductive physiology, emphasizing the importance of the rhythmic activity if the Leydig cells underpin male fertility.

Conflict of Interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Author Contributions

M.LJ.M.— data acquisition; data analysis and interpretation; figures drafting; revising manuscript critically for important intellectual content; S.A.A.—acquisition of the data; analysis and interpretation of the data; revising manuscript critically for important intellectual content; T.S.K.—the conception and design of the research; acquisition of the data; analysis and interpretation of the data; drafting the manuscript; All authors—approved the final version of the manuscript; agree to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

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