Stress-induced glucocorticoids alter the Leydig cells` timing and steroidogenesis-related systems

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Abstract

The study aimed to analyze the time-dependent consequences of stress on gene expression responsible for diurnal endocrine Leydig cell function connecting them to the glucocorticoid-signaling. In the first 24h after the stress event, a daily variation of blood corticosterone increased, and testosterone decreased; the testosterone/corticosterone were lowest at the end of the stress session overlapping with inhibition of Leydig cells' steroidogenesis-related genes (*Nr3c1*/GR, *Hsd3b1/2*, *Star*, *Cyp17a1*) and changed circadian activity of the clock genes (the increased *Bmal1*/BMAL1 and *Per1*/2/PER1 and decreased *Cry1* and *Rev-erba*). The glucocorticoid-treated rats showed a similar response. The principal-component-analysis (PCA) displayed an absence of significant differences between treatments especially on Per1 and Rev-erba, the findings confirmed by the in vivo blockade of the testicular glucocorticoid receptor (GR) during stress and ex vivo treatment of the Leydig cells with hydrocortisone and GR-blocker. In summary, stressful stimuli can entrain the clock in the Leydig cells through glucocorticoid-mediated communication.

Experimental design

Adult, three-month-old male Wistar rats were used for the experiments. Three in vivo experimental approaches were applied (1) to examine the effect of Immobilzation stress (IMO) on Leydig cell activity, IMO was applied from ZT0-3 followed by the expressional/ functional study in hours after IMO session (ZT3, ZT11, ZT17, and ZT23); (2) to prove that glucocorticoids mimic IMO effects on the Leydig cell function, rats were treated orally with Dexason (Galenika, SRB; 0.01mg/50 µL/100g BW) at ZT0 and the 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; (3) 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 (ZT0-3), and the effects were analyzed immediately after IMO. Control rats received 20 µL DMSO/testis at the same time as RU486 treated rats; (4) 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) was 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.

Results

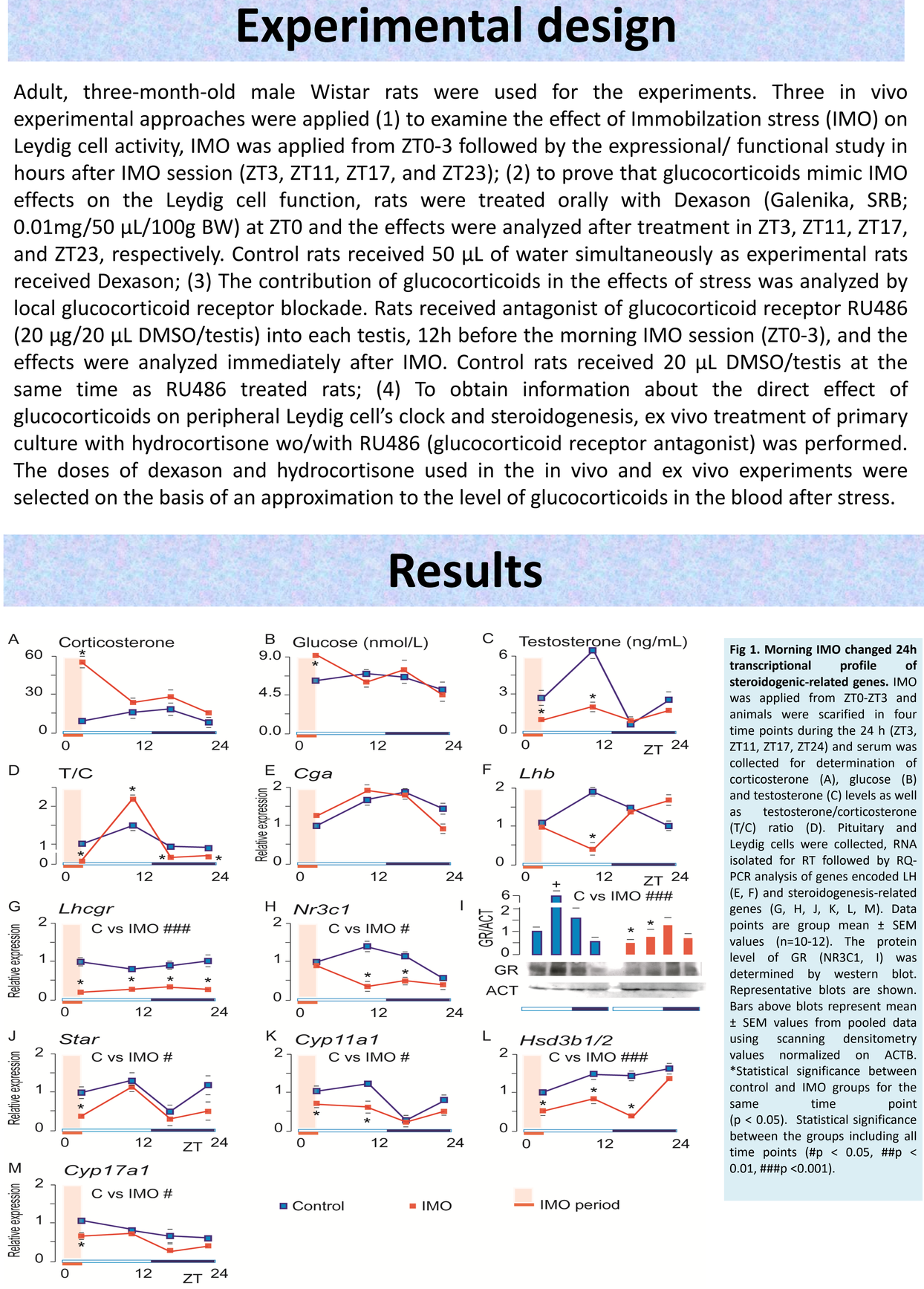

Fig 1. Morning IMO changed 24h transcriptional profile of steroidogenic-related genes. IMO was applied from ZT0-ZT3 and animals were scarified in four time points during the 24 h (ZT3, ZT11, ZT17, ZT24) and serum was collected for determination of corticosterone (A), glucose (B) and testosterone (C) levels as well as testosterone/corticosterone (T/C) ratio (D). Pituitary and Leydig cells were collected, RNA isolated for RT followed by RQ-PCR analysis of genes encoded LH (E, F) and steroidogenesis-related genes (G, H, J, K, L, M). Data points are group mean ± SEM values (n=10-12). The protein level of GR (NR3C1, I) 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 groups for the same time point ($p < 0.05$). Statistical significance between the groups including all time points (#$p < 0.05$, ##$p < 0.01$, ###$p <0.001$).

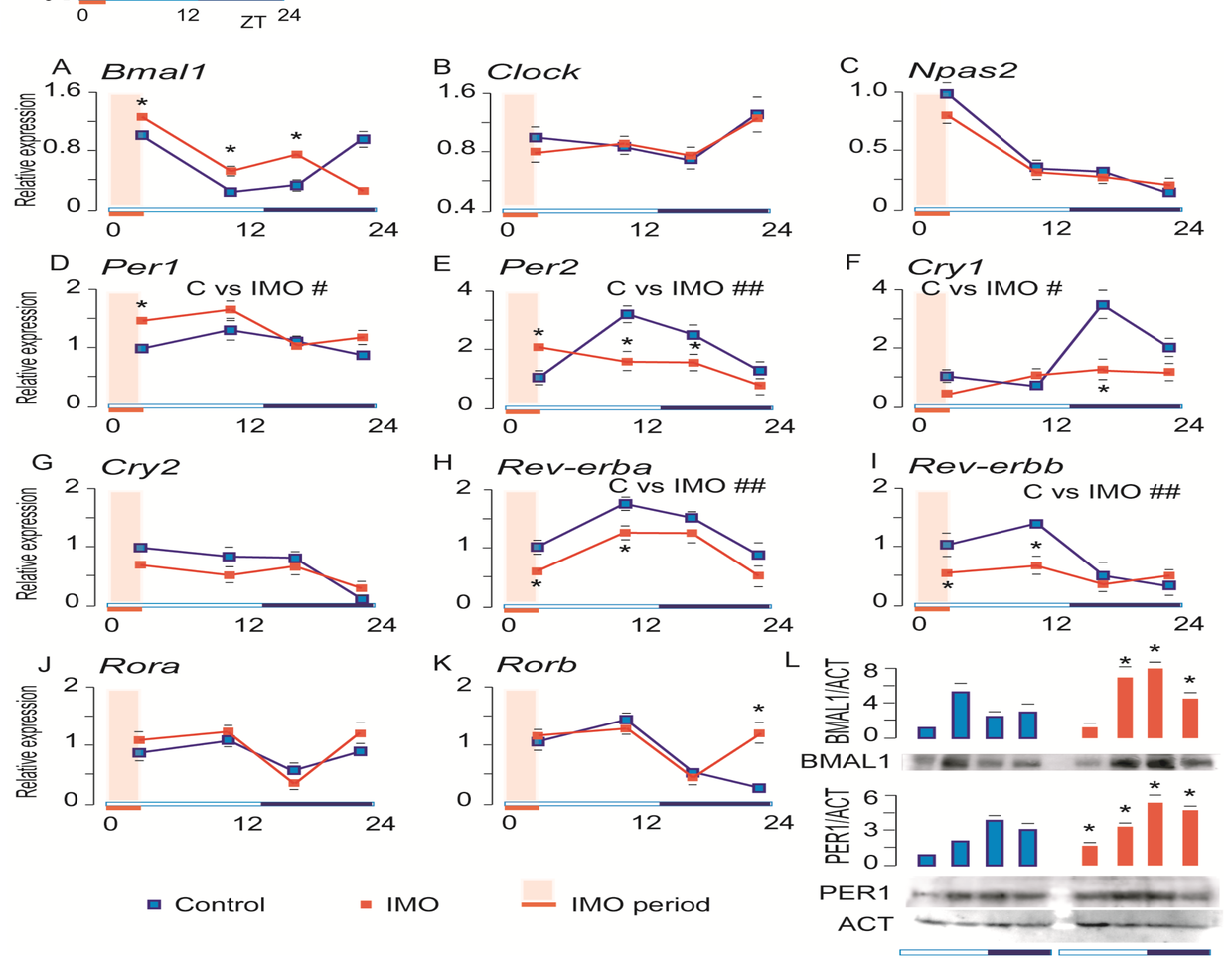

Fig 2. Morning IMO changed 24h transcriptional profile of clock genes. IMO **was** applied from ZT0-ZT3 and animals were sacrificed in four time points during the 24 h (ZT3, ZT11, ZT17, ZT24). Leydig cells were purified, RNA extracted, used for RT and consequently RQ-PCR analysis of clock genes expression (A-K). Data points are group mean ± SEM values (n = 10). The protein level of BMAL1 (L, upper panel) and PER1 (L, lower panel) 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 ($p < 0.05$). Statistical significance between the groups incl**ock genes.** IMO was uding all time points (#$p < 0.05$, ##$p < 0.01$, ###$p <0.001$).

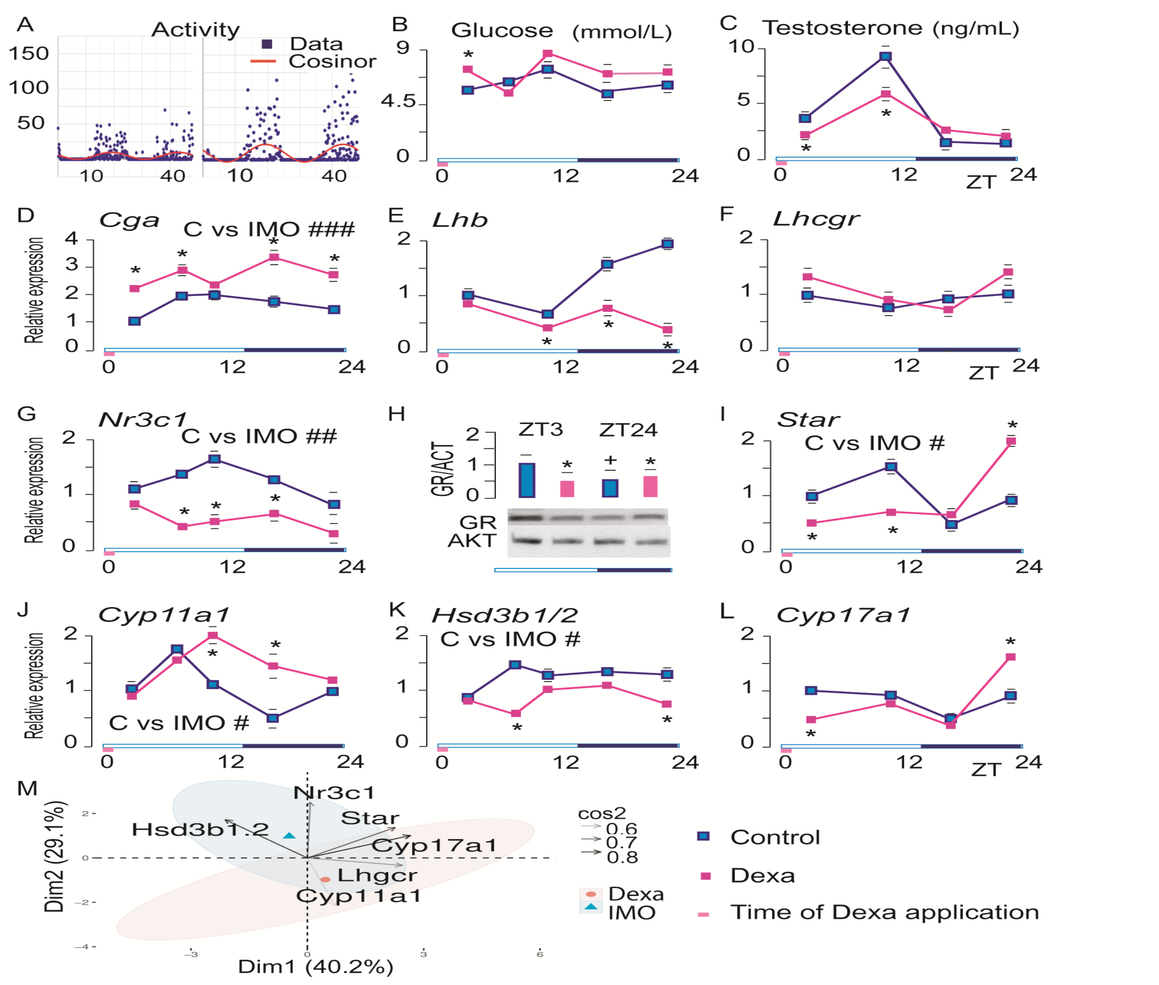

Fig 3. Treatment with glucocorticoids changed transcription rhythm of steroidogenic-related genes. *Wistar* rats were treated *per os* with Dexason (Dexa, 0.01mg/50µL/100g BW) at ZT0 and effects were analyzed after treatment in (ZT3, ZT7, ZT11, ZT117, ZT24). The voluntary rat's activity 48h before and 48h after Dexa application was recorded; the representative individual graf is shown (A). Serum was collected for determination of glucose (B) and testosterone (C) levels. Pituitary and Leydig cells were collected, RNA isolated for RT followed by RQ-PCR analysis of genes encoded LH (D,E) and steroidogenesis-related genes (F-L). Data points are group mean ± SEM (n=10). The protein level of GR (NR3C1, H) 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 Dexa groups for the same time point ($p < 0.05$); +Statistical significance with ZT3 ($p < 0.05$). Statistical significance between the groups including all time points (#$p < 0.05$, ##$p < 0.01$, ###$p <0.001$). ZT in this and all other figures is Zeitgeber time. PCA of steroidogenic-related genes in Leydig cells from IMO- and Dexa-treated groups (M); Dim1 and Dim2 represents the first two PC and % of retained variation. cos2 estimates the qualitative representation of variables.

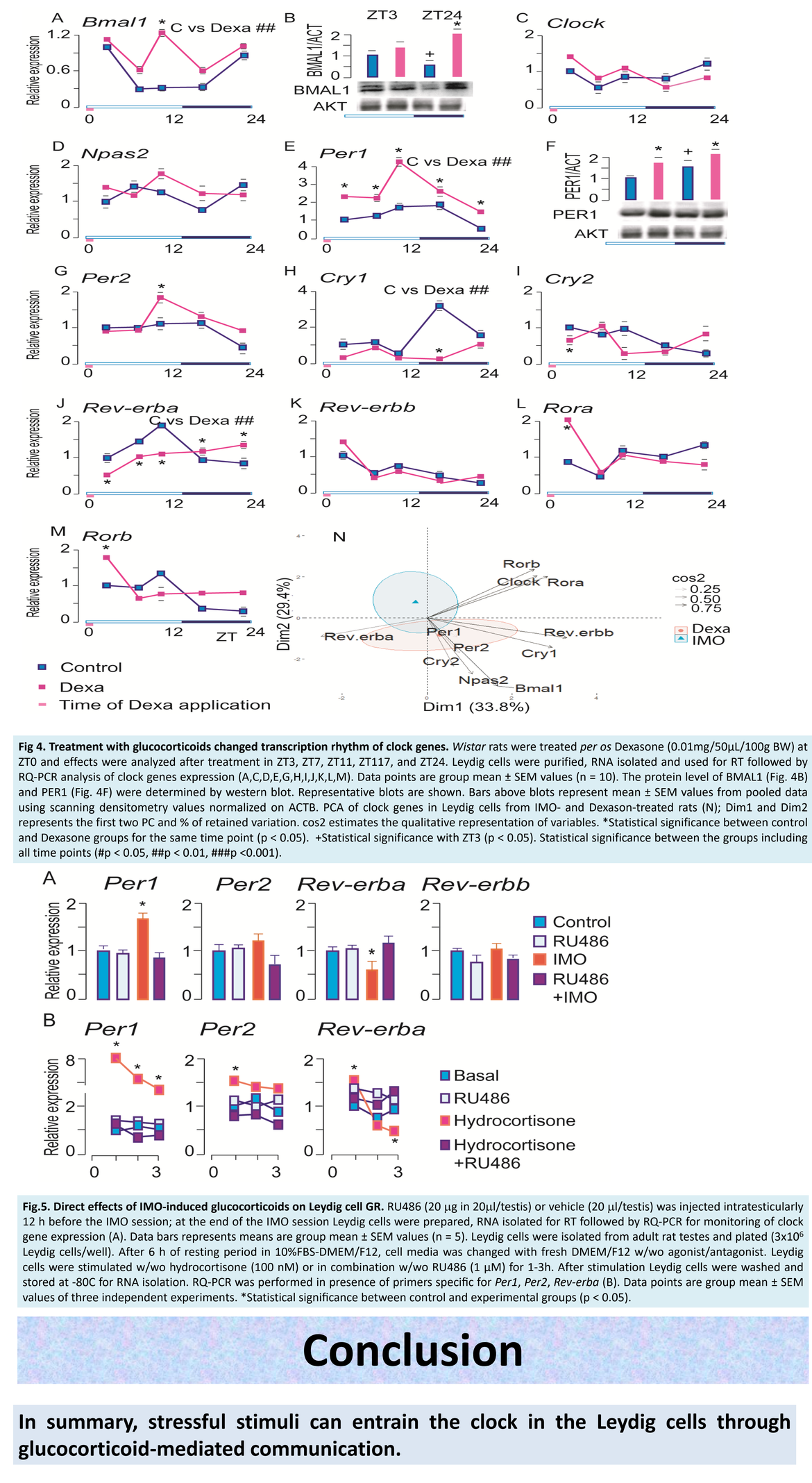

Fig 4. Treatment with glucocorticoids changed transcription rhythm of clock genes. *Wistar* rats were treated *per os* Dexasone (0.01mg/50µL/100g BW) at ZT0 and effects were analyzed after treatment in ZT3, ZT7, ZT11, ZT117, and ZT24. Leydig cells were purified, RNA isolated and used for RT followed by RQ-PCR analysis of clock genes expression (A,C,D,E,G,H,I,J,K,L,M). Data points are group mean ± SEM values (n = 10). The protein level of BMAL1 (Fig. 4B) and PER1 (Fig. 4F) 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. PCA of clock genes in Leydig cells from IMO- and Dexason-treated rats (N); Dim1 and Dim2 represents the first two PC and % of retained variation. cos2 estimates the qualitative representation of variables. *Statistical significance between control and Dexasone groups for the same time point ($p < 0.05$). +Statistical significance with ZT3 ($p < 0.05$). Statistical significance between the groups including all time points (#$p < 0.05$, ##$p < 0.01$, ###$p <0.001$).

Fig.5. 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 = 5). 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 w/wo agonist/antagonist. Leydig cells were stimulated w/wo hydrocortisone (100 nM) or in combination w/wo RU486 (1 µM) for 1-3h. After 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 ($p < 0.05$).

Conclusion

In summary, stressful stimuli can entrain the clock in the Leydig cells through glucocorticoid-mediated communication.

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