PERsuading nuclear receptors to dance the circadian rhythm

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ABSTRACT

The recurring light/dark cycle that has a period length of about 24 hours has been internalized in various organisms in the form of a circadian clock. This clock allows a precise orchestration of biochemical and physiological processes in the body thus improving performance. Recently, we found that the clock component PERIOD2 (PER2) can coordinate transcriptional regulation of metabolic, physiological, or behavioral pathways by interacting with nuclear receptors. PER2 appears to act as co-regulator of nuclear receptors linking clock function and transcriptional regulation at the level of protein-protein interactions. Here, we provide additional evidence for modulation of nuclear receptor dependent transcription by PER2 underscoring the broad implication of our finding. Taken together, our findings provide a base for the understanding of various disorders including mood disorders that have their roots in a temporal deregulation of basic metabolic processes.

INTRODUCTION

Circadian rhythms are oscillations that recur daily and are intrinsic to a biological system because they continue in the absence of external inputs. To sustain such 24-hour rhythms an autonomous clock mechanism is working in every body cell.[1] Since these individually ticking cellular clocks do not have precisely the same period length, they need to be synchronized to each other for the generation of a coherent systemic circadian rhythm. This rhythm is useful for the organism to prepare itself for daily recurring events such as feeding and sleeping. By the same token, it allows an organism to temporally separate anabolic and catabolic processes and improve performance. Hence, coordination of cellular clocks is key to systemic function. Furthermore, keeping the circadian system in resonance with the environment (e.g. day-night cycle) optimizes biological systems in order to allow them to maximally profit from food availability and geophysical properties such as light.

The mammalian circadian oscillator is constituted of an auto-regulatory transcriptional-translational feedback loop.[2] Two transcriptional activators BMAL1 and CLOCK (or NPAS2 in the brain) bind to E-box elements present on the promoters of other clock genes (or clock controlled genes) thereby supporting activation of their transcription. Two classes of transcriptional repressors that are activated this way act back on the transcriptional activators either directly (REV-ERBα) by repressing the *Bmal1* promoter, or indirectly via inhibition of the activity of the BMAL1/CLOCK complex (PERIOD and CRYPTOCHROME proteins). Various proteins including kinases, phosphatases and ubiquinating enzymes modulate the half-life and/or efficiency of nuclear transport of clock components, which ultimately leads to a roughly 24-hour rhythm of transcriptional activation of clock genes in the cell nucleus. It is evident that such a mechanism needs coordination between the activator and repressor components. Such coordination may be mediated by PERIOD2 (PER2), which can interact on one hand with the BMAL1/CLOCK complex influencing its activity[3,4] and on the other hand with nuclear receptors modulating *Bmal1* gene

expression such as REV-ERBα and PPARα.[5] Furthermore, coordination between the clock mechanism and BMAL1/CLOCK mediated and/or nuclear receptor mediated transcription of genes is of great importance for generation of coherent systemic rhythms of metabolic gene expression. This coordination may again be mediated by PER2 in its capacity to interact with both nuclear receptors and clock proteins.[5]

Interaction potential of the PER2 protein

The PER proteins bear several protein-protein interaction domains that allow them to interact with a multitude of proteins such as BMAL1/CLOCK and CRY1.[3,6] These interactions may form the basis for the molecular oscillator. Previously, we found two additional classes of interaction motifs in the PER proteins: the CoRNR and LXXLL motifs,[7] which are characteristic for interactions of nuclear receptors with their co-regulators.[8,9] While the putative CoRNR boxes are well conserved between the different isoforms of PER proteins, they differ in their potential LXXLL motifs. Based on these observations, we examined whether the PER2 protein could physically interact with nuclear receptors.[5] Nuclear receptors are potent transcriptional regulators and control the expression of multiple genes involved in various processes such as development, reproduction and metabolism.[10,11] In an *in vitro* assay, we supplemented mouse NIH 3T3 fibroblasts with expression vectors for V5-tagged PER2 and for HA-tagged nuclear receptors to monitor physical interactions by co-immunoprecipitation. Our selection of nuclear receptors included receptors that are either known to influence the circadian oscillator or are expressed in a similar phase than the PER proteins in liver.[10] We observed *in vitro* physical interactions of PER2 with several nuclear receptors such as REV-ERBα, PPARα, NURR1, HNF4α and TRα. These interactions depended on the presence of LXXLL-like sequences in PER2. Taken together, PER2 has the

capacity to physically interact with a selection of nuclear receptors, extending the regulatory potential of PER2.

Target gene regulation by PER2

Similarly to our *in vitro* results, PER2 was found to form complexes with the nuclear receptors PPARα and REV-ERBα in liver nuclei. Both nuclear receptors are known to control the rhythmic expression of the clock gene *Bmal1* in the liver. The transcriptional repressor REV-ERBα and the activator PPARα bind to two different nuclear response elements (NREs) in the *Bmal1* promoter in different time windows of the circadian cycle. The physical interaction of PER2 with these two transcriptional regulators suggested importance of these interactions for *Bmal1* gene regulation in the circadian oscillator. Using chromatin immunoprecipitation (ChIP), we tested for the presence of PER2 at the promoter of the *Bmal1* gene. We found PER2 bound at the *Bmal1* promoter region in a REV-ERBα dependent manner. Additionally, PER2 binding was observed at the upstream regulatory region of the *Bmal1* gene that bears binding sites for PPARα.[12] In this situation, PER2 binding overlapped with PPARα binding and was observed during transcriptional activation of the *Bmal1* gene.

To monitor the effect of PER2 on the regulation of the *Bmal1* gene in the liver, we measured mRNA accumulation of this gene in the liver of mice with different genetic backgrounds. *Bmal1* mRNA accumulation in the liver of $Per2^{Brdm1}$ mice was reduced with blunted amplitude. Furthermore, in mice lacking functional *Per2* and *Rev-Erbα*, *Bmal1* expression was constitutive at the level of maximal expression in $Per2^{Brdm1}$ mice. These results suggest that PER2 can modulate *Bmal1* transcription and function as co-regulator of nuclear receptor-mediated transcription. In this manner, PER2 contributes to the high-amplitude oscillation of *Bmal1* mRNA accumulation in the liver and also in the suprachiasmatic nuclei (SCN). Thus, PER2 may be involved in the coupling of the core and

stabilizing loop of the oscillator. Here we provide additional evidence underscoring the importance of PER2 in regulating circadian gene expression mediated by nuclear receptors.

RESULTS and DISCUSSION

Function of PER2 in the stabilizing loop of the circadian oscillator in NIH 3T3 cells

Our previous results suggest that in the liver circadian expression of the *Bmal1* gene is regulated by the alternate action of two nuclear receptors, REV-ERBα and PPARα. Both can interact with PER2 to modulate *Bmal1* expression. Here, we analyzed the analogous stabilizing loop of the clock in synchronized NIH 3T3 mouse fibroblasts (Fig. 1). Tissue culture cells offer the advantage of a free-running oscillator not affected by external timing cues.[13] ChIP analysis revealed the presence of two nuclear receptors on the *Bmal1* promoter (Fig. 1A). RORα and REV-ERBα bound to the regulatory region in the phase of transcriptional activation and repression of *Bmal1*, respectively (Fig. 1A, 1B). Similar to the situation in the liver, PER2 binding to this promoter region paralleled binding of REV-ERBα. Therefore, PER2 may modulate the repressive potential of REV-ERBα. Interestingly, the peaks of *Per2* and *Rev-Erbα* mRNA accumulation were separated by roughly 8 hours in synchronized cells (Fig. 1B). This was also reflected by a separation of the corresponding protein peaks by about the same interval (Fig. 1C). Consequently, there is only a small temporal overlap between both proteins. This time window corresponds to the moment when both proteins are present at the *Bmal1* promoter region. As a speculation, binding to the regulatory region is driven by post-translational modifications in either of the two proteins. The interaction of both proteins may shape the accumulation of *Bmal1,* because PER2 modulates REV-ERBα repressive activity.

In NIH 3T3 fibroblasts, binding of RORα seems to be independent of PER2 (Fig. 1A). The alternate binding of RORα and REV-ERBα to the same regulatory element of the *Bmal1* gene is in agreement with previous studies.[14,15] According to this regulatory model, both nuclear receptors may compete for the same DNA binding element. It can be speculated that PER2 might influence this competition and therefore modulates the balance between activation and repression of *Bmal1*. However, this remains to be elucidated. REV-ERBα recruits the corepressor N-CoR1 for its repressive function on the *Bmal1* promoter.[16] Since both factors are present on the *Bmal1* gene *in vivo*, competition between PER2 and N-CoR1 could be envisaged. REV-ERBα and PER2 potentially bind heme.[17-19] REV-ERBβ is binding heme as well and switches ligand in a redox-dependent manner with CO- and NO-induced ligand displacement.[20] Thus, one can speculate that a similar mechanism applies to REV-ERBα and PER2. Hence, REV-ERBα and PER2 may modulate cellular functions in a heme-, redox- or gas-regulated manner thereby integrating metabolic state for transcriptional regulation.

Taken together, our data suggest that PER2 acts as co-regulator in the stabilizing loop of the circadian oscillator in NIH 3T3 fibroblasts. Thus, the function of PER2 in this pathway may occur in essentially all tissue clocks.

Distinct influences of nuclear receptors on the circadian oscillator

Previously, the influence of PPARα and REV-ERBα on two different circadian reporter genes was analyzed.[5] PPARα together with its heterodimerisation partner RXRα did not affect expression of either of the two reporter genes. By contrast, co-transfection of REV-ERBα repressed activity of the *Rev-Erbα* and *Bmal1* luciferase reporter vectors in NIH 3T3 fibroblasts (Fig. 2A and Schmutz et al.[5]). Here we extend the analysis using other nuclear receptors that either can be immunoprecipitated with PER2 *in vitro* (NURR1, HNF4α, TRα)

or can not be immunoprecipitated with PER2 (RORα, ERα, RXRα). Based on their action on the reporter constructs *Rev-Erbα luc* and *Bmal1 luc* in NIH 3T3 cells, we could define three groups of nuclear receptors (Fig. 2). Group I represented by REV-ERBα and NURR1 repressed the activity of either the *Rev-Erbα* or *Bmal1* luciferase reporter gene (Fig. 2A), group II represented by RORα and ERα activated at least one reporter gene (Fig. 2B), and group III represented by HNF4α, RXRα and TRα did not have a measurable impact on their activity (Fig. 2C). Hence, not only the standard nuclear receptors involved in the stabilizing loop (RORα, REV-ERBα, and PPARα) are capable to feed back on clock gene expression. Other nuclear receptors such as NURR1 and ERα may directly or indirectly fulfill a similar function.

To monitor the impact of PER2 on these different groups of nuclear receptors, we performed co-transfection experiments (Fig. 3). Co-transfection with increasing amounts of PER2 elevated specifically the level of *Bmal1*-driven luciferase activity as observed previously.[5] For co-transfection experiments with the additional nuclear receptors, we kept the amount of *Per2-V5* expression vector constant. The nuclear receptor NURR1 alone decreased *Bmal1 luc* activity in a dose-dependent fashion (Fig. 2A, 3A). Co-transfection with PER2 modulated this effect. NURR1-mediated repression of *Bmal1 luc* activity was attenuated in the trough of cycling (Fig. 3A). This resembled a similar experiment performed previously with PER2 and REV-ERBα (suppl. Fig. 7 in Schmutz et al.[5]). Hence, NURR1 and REV-ERBα both act as transcriptional repressors of *Bmal1* reporter activity and their repressive potential is modulated by PER2.

The transcriptional activator PPARα alone does not change *Bmal1* reporter activity but shows synergism together with PER2 in the presence of RXRα.[5] By contrast, co-transfection of RORα did not affect the action of PER2 on *Bmal1* (Fig. 3B). This could indicate that action of RORα on this gene is independent of PER2 as it was already suggested

by the ChIP results (Fig. 1A) or that an unknown heterodimerization partner for RORα is necessary. Additionally, HNF4α did not collaborate with PER2 on *Bmal1* reporter gene expression (data not shown). Together with the results from Fig. 2C, this indicates that HNF4α does not impinge on the stabilizing loop of the circadian oscillator but may use PER2 to coordinate output functions as shown for its PER2 supported action e.g. on the *Hnf1α* promoter.[5] Of interest here is that *Hnf4α* is not expressed in a circadian manner in the liver compared to *Rev-Erbα*, *Pparα* and *Nurr1*.[10] This indicates that PER2 confers circadian information to this non-circadian nuclear receptor. Taken together, PER2 modulates the expression of clock output genes such as *Hnf1α* (via HNF4α) and other yet to be determined metabolic genes by interaction with nuclear receptors. Furthermore, PER2 can coordinate the circadian feedback loop by physical interaction with nuclear receptors (REV-ERBα, PPARα, and NURR1).

In this study, we provide evidence that the nuclear receptor NURR1 is modulated in its activity by PER2 in NIH3T3 cells. This nuclear receptor plays an essential role in the generation and maintenance of dopaminergic neurons in the brain.[21] It is involved in the regulation of genes coding for aromatic amino acid decarboxylases, tyrosine hydroxylase (TH), and the dopamine transporter[22] leading to the notion that it plays an important role in dopaminergic nerve function and diseases associated with the dopaminergic system. In agreement with this notion it appears that NURR1 is involved in depression-like behavior in mice[23,24] and its expression is reduced in the brain of schizophrenic and bipolar patients.[25] Interestingly, mice with a mutation in the clock gene *Per2* show alterations in mood related behaviors associated with elevated dopamine levels in the mesolimbic dopaminergic system.[26] In these animals, TH expression in the nucleus accumbens is not diurnal in contrast to wild type controls.[27] This suggests that NURR1-mediated transcription of TH is modulated

by PER2, which no longer occurs in *Per2* mutant mice. However, regulation of the TH promoter by NURR1 and PER2 needs to be investigated in the future to test this hypothesis.

CONCLUSIONS

Nuclear receptors play central roles in development, physiology and metabolism. Their action can be modulated via small ligands and co-regulator proteins. PER2 appears to have functional characteristics of the latter and may impinge time dependent functions on nuclear receptors. Therefore, nuclear receptors that do not display a circadian pattern of expression may still be modulated in their activities in a time dependent fashion. This links functions of nuclear receptors in the central nervous system, metabolism, energy homeostasis, xenobiotic mechanisms, steroidogenesis and reproduction with the circadian clock. Therefore, diseases such as mood related disorders, hypertension, inflammatory diseases, diabetes, obesity and cancer are related to clock function.

MATERIALS and METHODS

Chromatin Immunoprecipitation (ChIP) from NIH 3T3 cells

NIH 3T3 cells were grown to confluency in DMEM High Glucose (Sigma) supplemented with 10 % fetal calf serum and 100 U/ml penicillin/streptomycin (Amimed) on 15 cm plates. After synchronization with 100 nM dexamethasone,[28] the cells were kept in DMEM High Glucose (Sigma) supplemented with 0.5 % fetal calf serum until harvest. At the indicated time points, cells were fixed with 1 % formaldehyde in 1x PBS for 10 min at 37°C. Cell nuclei were extracted according to the cell fractionation protocol, washed once with 1 mM EDTA, 1 mM EGTA, 10 mM Hepes pH 6.8, 0.5 mM DTT, 200 mM NaCl, and resuspended in 400 µl of 20 mM Tris-HCl pH 7.5, 150 mM NaCl, 2 mM EDTA supplemented with 1 % SDS. Sonication, dilution, immunoprecipitation, washing conditions and detection of the mouse *Bmal1* promoter region (+50) were as described for ChIP from mouse liver nuclei in Schmutz et al.[5]

RNA quantification from NIH 3T3 cells

Total RNA was extracted from cells using RNA-Bee (AMS Biotechnology) according to the manufacturer. RNA was further purified by phenol:chloroform:isoamylalcohol extraction and ethanol precipitation. ssDNA complementary to the RNA was synthesized with Superscript II (Life Technology Corporation) according to the manufacturer starting from hybridized random hexamer primers. mRNA levels were quantified by Taqman real-time PCR. PCR probes have been described.[5] After normalization to *Gapdh* mRNA accumulation, relative mRNA levels were assessed by defining the highest value of each experiment as 1.

Cellular fractionation and Western blot analysis

For cellular fractionation, cells were broken by swelling the cells in 100 mM Tris-HCl pH 8.8/ 10 mM DTT and subsequent hypotonic lysis (10 mM EDTA, 1 mM EGTA, 10 mM

Hepes pH 6.8, 0.2 % Triton-X 100, 0.5 mM DTT, protease inhibitor cocktail, 0.2 mM NaV, 5 mM NaF). After centrifugation for 5 minutes at 2,500 g and 4°C, the supernatant was stored as cytosolic fraction. Nuclear extracts were prepared from purified nuclei according to the NUN procedure [29]. Briefly, nuclei were re-suspended in 1x NDB (100 mM KCl, 0.2 mM EDTA, 20 % glycerol, 20 mM Hepes pH 7.6, 2 mM DTT) and an equal volume of 2x NUN (600 mM NaCl, 2 M Urea, 2 % NP-40, 50 mM Hepes pH 7.6) was added. After mixing, the samples were incubated on ice for 30 min and centrifuged for 30 min at 16,000 g and 4°C. The supernatant was stored as nuclear extract. Western blot analysis was performed according to Schmutz et al.[5]

Real-time bioluminescence monitoring

Expression vectors and luciferase reporter vectors were as described in Schmutz et al.[5] For real-time bioluminescence monitoring, NIH 3T3 mouse fibroblasts cultured in DMEM High Glucose (Sigma-Aldrich) supplemented with 10 % fetal calf serum and 100 U/ml penicillin/streptomycin (Amimed) were split onto 3.5 cm Ø Falcon dishes. Proliferating cells were transfected with appropriate luciferase reporter vectors (1.8 µg), the indicated amounts of expression vectors, and *pCMV-SEAP* (0.1 µg) for normalization. 48 hours after transfection, cells were synchronized by addition of DMEM containing 100 nM dexamethasone.[28] After 20 minutes, the medium was changed to phenol red-free DMEM supplemented with 0.1 mM luciferin and 5 % FCS. Bioluminescence was continuously monitored for 4 to 5 days using a LumiCycle apparatus (Actimetrics). Bioluminescence recordings were analyzed using LumiCycle analysis software. Raw data were normalized to the secreted alkaline phosphatase activity (SEAP reporter gene assay, Roche) in a culture medium sample taken before synchronization.

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FIGURE LEGENDS

Figure 1: PER2 binding to the *Bmal1* promoter in NIH 3T3 cells

NIH 3T3 mouse fibroblasts were synchronized by dexamethasone shock, and analyzed at 4 hrs intervals starting 12 hrs after the shock. (A) Chromatin Immunoprecipitation (ChIP) with αREV-ERBα, αRORα and αPER2 using specific Taqman probes for the *Bmal1* promoter region (mean ± SD from three independent experiments). (B) *Bmal1*, *Per2* and *Rev-Erbα* mRNA levels were quantified by Taqman real-time PCR. Plotted values are the mean values ± SD from three independent experiments. (C) Nuclear protein extracts were analyzed by immunoblotting using the indicated antibodies. Dex: dexamethasone.

Figure 2: Impact of nuclear receptors on clock gene expression.

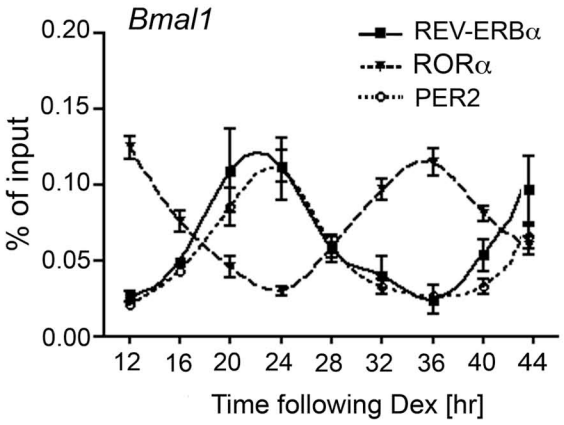
(A-C) NIH 3T3 cells were transfected with either *Rev-Erbα* luciferase reporter vector (left panels) or *Bmal1* luciferase reporter vector (right panels) either alone or together with the indicated expression vectors. Amounts of expression vectors were adjusted according to the expression level of the given protein in NIH 3T3 cells. The amounts used are indicated. 48 hrs after transfection, cells were synchronized with dexamethasone and luciferase activity was monitored in real-time using a LumiCycle apparatus. Data are plotted as mean + SD (representative experiment out of three independent experiments).

Figure 3: PER2 modulates the activity of nuclear receptors

NIH 3T3 cells were transfected with a *Bmal1* luciferase reporter vector either alone or together with increasing amounts of *HA-nuclear receptor* expression vector (grey) or a constant amount of *Per2-V5* expression vector (blue). Dashed lines (green) represent co-transfection of the same increasing amounts of *HA-nuclear receptor* expression vector together with the constant amount of *Per2-V5* expression vector. Amounts of *HA-nuclear receptor* expression vectors were adjusted according to the expression level of the given

protein in NIH 3T3 cells. (A) *HA-Nurr1* was cotransfected with *Per2-V5*. (B) *HA-Ror*α was cotransfected with *Per2-V5*. Cells were synchronized by a dexamethasone shock and luciferase activity was recorded using a LumiCycle apparatus. Data are plotted as mean only (representative experiment out of three independent experiments).

A


Bmal1
REV-ERBα
RORα
PER2
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0.10
0.05
0.00
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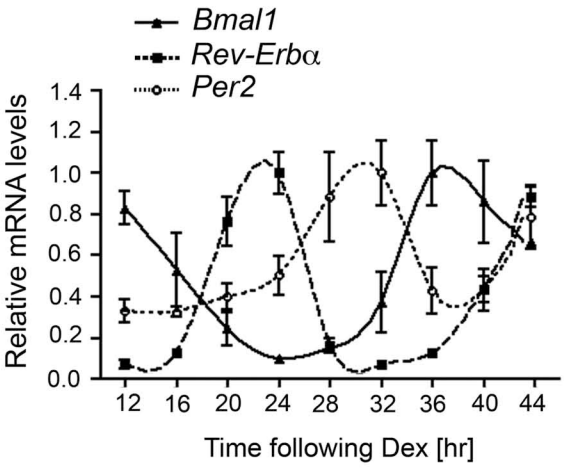
Time following Dex [hr]

B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Rev-Erbα
Per2
Relative mRNA levels
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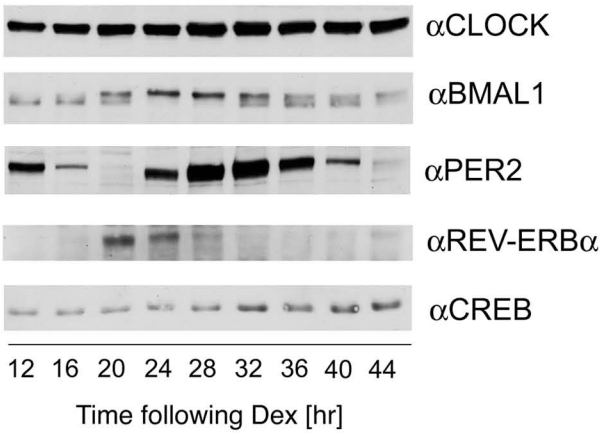
0.8
0.6
0.4
0.2
0.0
12 16 20 24 28 32 36 40 44
Time following Dex [hr]

C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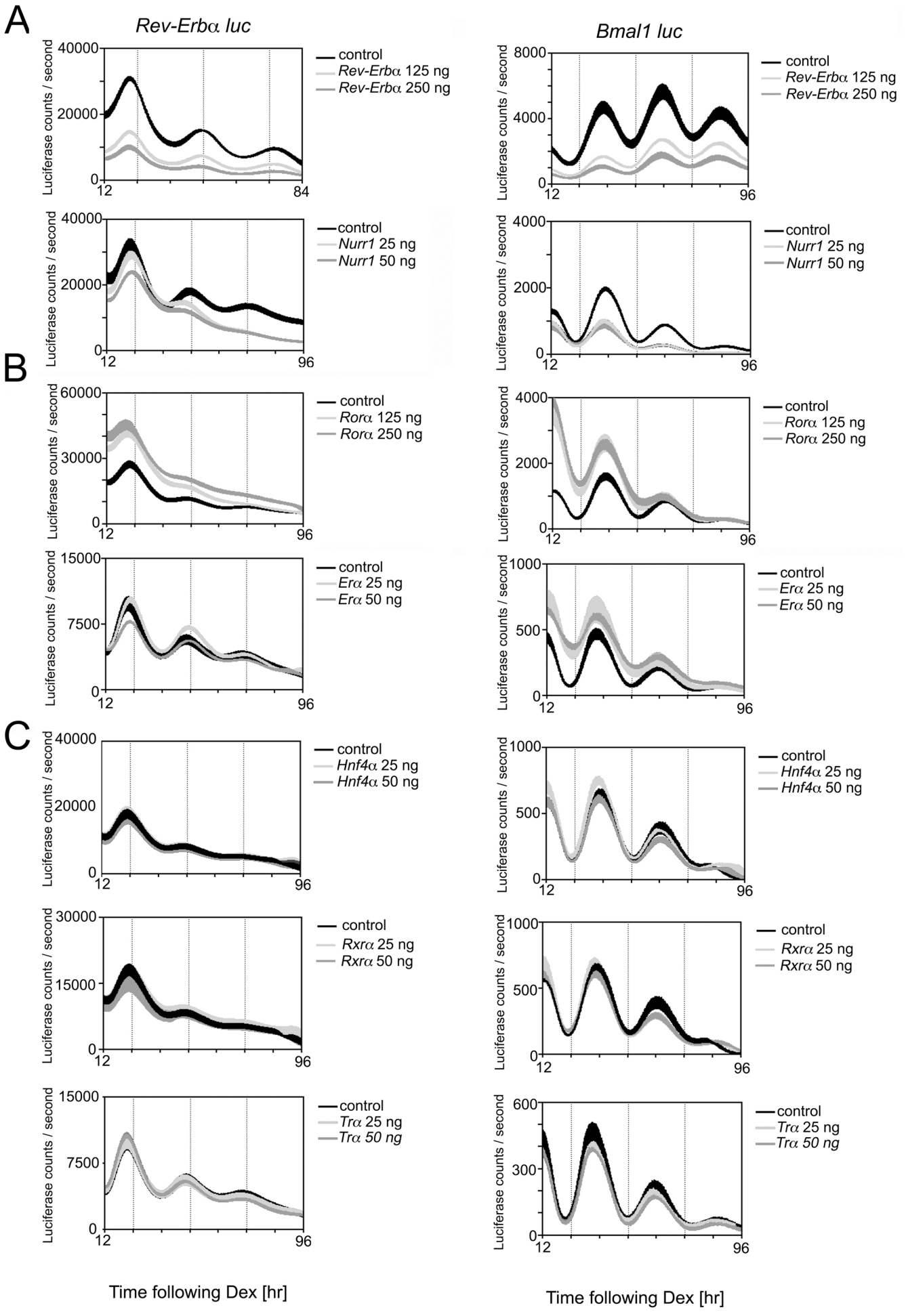
Time following Dex [hr]

Fig. 1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Bmal1 luc
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Nurr1 25 ng
Nurr1 50 ng
B
Rorα 125 ng
Rorα 250 ng
Erα 25 ng
Erα 50 ng
C
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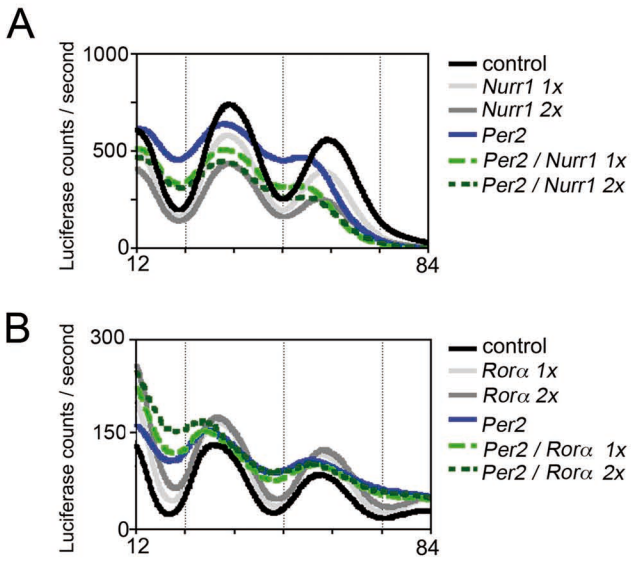
Trα 50 ng
Time following Dex [hr]

Fig. 2


A
Luciferase counts / second
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500
0
12
84
control
Nurr1 1x
Nurr1 2x
Per2
Per2 / Nurr1 1x
Per2 / Nurr1 2x
B
Luciferase counts / second
300
150
0
12
84
control
Rorα 1x
Rorα 2x
Per2
Per2 / Rorα 1x
Per2 / Rorα 2x

Fig. 3