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Retinoic acid receptors move in time to the clock in the hippocampus. Effect of a vitamin A-deficient diet

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Abstract

An endogenous time-keeping mechanism controls circadian biological rhythms in mammals. Previously, we showed that the vitamin A deficiency modifies clock BMAL1 and PER1 as well as BDNF and Neurogranin daily rhythmicity in the rat hippocampus, when animals are maintained under 12h-light:12h-dark conditions. Retinoic acid nuclear receptors, RARs and RXRs, have been detected in the same brain area. Our objectives were 1) to analyze whether RAR α , RAR β and RXR β exhibit a circadian variation in the rat hippocampus, 2) to investigate the effect of a vitamin A-deficient diet on the circadian expression of BMAL1, PER1 and retinoic acid receptors (RARs and RXR β) genes. Holtzman male rats from control and vitamin A-deficient groups were maintained under 12h-light:12h-dark or 12h-dark:12h-dark during the last week of treatment. RAR α , RAR β , RXR β , BMAL1 and PER1 transcript and protein levels were determined in hippocampus samples isolated every 4h in a 24h period. Regulatory regions of RARs and RXR β genes were scanned for clock-responsive sites while BMAL1 and PER1 promoters were analyzed for RAREs and RXREs. E-box and RORE sites were found on regulatory regions of retinoid receptors genes, which display an endogenously-controlled circadian expression in the rat hippocampus. Those temporal profiles were modified when animals were fed with a vitamin A-deficient diet. Similarly, the nutritional vitamin A deficiency phase shifted BMAL1 and abolished PER1 circadian expression at both, mRNA and protein levels. Our data suggests that the vitamin A deficiency may affect the circadian expression in the hippocampus by modifying the rhythmic profiles of retinoic acid receptors.

Keywords

vitamin A; RAR; RXR; circadian rhythm; BMAL1; PER; hippocampus

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INTRODUCTION

Biological rhythms provide a mechanism by which organisms are kept in sync with their oscillating environment, being part of a predictive-adaptive strategy, critical to the survival of the species [1,2,3]. Learning and memory processes underlie animals' predictive and behavioral adaptation to temporal fluctuations in the environment, such as light/dark period, food and water availability, social cues or risk of predation [4,5]. Endogenous rhythms that oscillate with a 24-h periodicity, are called circadian (from Latin: *circa*, about; and *dies*, day) rhythms. In mammals, the more evident circadian rhythms include, among others, the sleep/wake and activity/rest cycles [6,7] however, a wide-ranging of physiological processes and metabolic parameters also display circadian oscillations. Nowadays, we know these rhythms are controlled by a central, master clock in the suprachiasmatic nucleus (SCN) [8,9,10]. The master clock in the SCN entrains and synchronizes the multiple peripheral clocks located in most, if not all, of tissues and cells in the body, either by neural and/or humoral signals [11,12,13]. The cellular molecular machinery of the circadian oscillator is composed of two interacting transcription/translation-based feedback loops, a positive and a negative one. The positive circuit consists of the heterodimer BMAL1:CLOCK (from *Brain and Muscle ARNT Like protein 1: Circadian Locomotor Output Cycles Kaput* protein) which binds clock-responsive, E-box, sites (CANNTG) on its target genes promoters [14,15,16]. Thus, BMAL1:CLOCK activates the transcription of other clock genes, such as Periods (Per1, Per2, and Per3) and Cryptochroms (Cry1 and Cry2), as well as other clock-controlled target genes [9,13]. After translation, the PER and CRY proteins form cytoplasmic complexes which are phosphorylated by Casein kinase 1 ϵ (CK1 ϵ) and CK1 δ enzymes [17,18]. Afterwards, CRY-PER-P complexes translocate to the nucleus where they initiate the negative circuit. CRY:PER-P complexes interfere with the BMAL1:CLOCK-mediated transcriptional activation of clock and clock-controlled genes, thus, reducing the amplitude of their circadian oscillating expression, in a general mechanism conserved in many organisms [9,13,17,19]. Other components which complete the molecular clock machinery are the nuclear receptors and transcription factors ROR (from *retinoic acid receptor-related orphan receptor*) and REV-ERB. These act in concert to activate (ROR), or repress (REV-ERB), BMAL1 transcription. Thus, by competing for the same response element in the BMAL1 promoter, they drive the circadian rhythmic expression of BMAL1 [20, 21, 22, 23].

External environmental factors serve as indicators of the astronomical (solar) time. They are called *zeitgebers*, literally from German: time-givers. The best known *zeitgeber* is the light-dark cycle. The environmental information accedes through the retina and the retinohypothalamic tract to the SNC [24,25]. Other *zeitgebers* include temperature, tides, social contact and food availability, among others [26,27,28]. Although it is known feeding/starving cycles are dominant *zeitgebers* for many peripheral clocks [11, 29, 30], the mechanisms by which feeding synchronizes these clocks are still poorly understood. Food metabolites such as glucose and/or feeding-related hormones such as glucagon and insulin have been propose as likely candidates for phase setting of peripheral clocks [11]. Indeed, as suggested by Schibler and Sassone-Corsi [11], given the multitude of signals able to generate circadian rhythms in tissue culture cells, it is likely that a variety of different pathways are also used in vivo to synchronize peripheral clocks. We wonder whether Vitamin A, a micronutrient involved in a wide spectrum of biological functions, could participate in the regulation of circadian rhythms in peripheral clocks. It is known that much of the action of vitamin A is mediated via specific nuclear receptors, retinoic acid (RA) receptors (RARs, α , β , and γ) and retinoid X receptors (RXRs, α , β and γ). While RARs bind both all-trans-RA (ATRA) and 9cis-RA, RXRs only bind 9cis-RA to exert their action as transcription factors on retinoid-responsive target genes [31,32,33]. Some evidence raises the possibility that, through its specific nuclear receptors, vitamin A may act as a clock

activity regulator, for example, by modulating the BMAL1/CLOCK complex binding to DNA [34].

In the last decade, a role for a vitamin A oxidized derivative, the RA, has also been established in phenomena related to higher cognitive functions in the brain of adult mice [35, 36, 37]. While the RAR α receptor is widely distributed in the brain, several authors have observed a distribution and specific localization of the RAR β , RAR γ , RXR β and RXR γ receptors in the mouse hippocampus [38,39]. It is well established that the hippocampus is critically involved in memory formation and time-of-day effects on learning and memory have been observed in human, non-human primates and rats [40,41,42,43].

Previously, we showed that vitamin A deficiency (VAD) decreased basal RXR α and/or RXR β expression in different tissues [44,45,46]. In this study, our aims were: 1) to analyze the RAR α , RAR β and RXR β expression, at mRNA and protein levels, throughout a 24-h period in the hippocampus of rats maintained under light:dark and constant darkness conditions, 2) to investigate the effect of a vitamin A-deficient diet on the circadian expression of clock (BMAL1 and PER1) and retinoic acid receptors (RAR α , RAR β , RXR β) genes and proteins in the hippocampus, a peripheral oscillator with a fundamental role in cognitive functions.

This would be, at least at our knowledge, the first published report on the circadian rhythmicity of retinoic acid nuclear receptors (RAR α , RAR β and RXR β) and the effects of the nutritional vitamin A deficiency on those circadian patterns in the hippocampus.

MATERIALS AND METHODS

Animals and Diets

Holtzman rats were weaned at 21 days and immediately assigned to either the experimental diet, devoid of vitamin A [vitamin A-deficient (VAD) group] or the same diet with 4000 IU of vitamin A per Kg of diet [control (Co) group] during 3 months. Rats were maintained in a 21-23°C controlled environment with free access to food and water throughout the entire experimental period. Thirty six animals from each group were maintained under 12h-light: 12h-dark (LD condition) or 12h-dark:12h-dark during the last week (DD condition). Diets were prepared according to the AIN-93 for laboratory rodents [47]. Both, vitamin A-deficient and control diets had the following composition (g/Kg): 397,5 cornstarch, 100 sucrose, 132 dextrinized cornstarch, 200 lactalbumin, 70 soybean oil, 50 cellulose fiber, 35 AIN-93 mineral mix, 10 AIN-93 vitamin mix (devoided of vitamin A for the vitamin A-deficient diet), 3 L-cystine, 2.5 choline bitartrate, and 0,014 tert-butylhydroquinone. After the treatment period, six rats from each group (Co and VAD) were sacrificed every 4 hours during a 24-h period, at the zeitgeber times (ZT): ZT2, ZT6, ZT10, ZT14, ZT18 and ZT22 (with ZT0 when light is on) for rats in LD condition (n= 36), and at the circadian times (CT): CT2, CT6, CT10, CT14, CT18 and CT22 (with CT0 when subjective day starts) for rats in DD condition (n=36). In the last case, rats were killed under dim red light to avoid acute effects of light. All experiments were repeated at least twice. They were conducted in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals (NIH Publications No.80-23) and the National University of San Luis Committee's Guidelines for the Care and Use of Experimental Animals.

Hippocampus dissection

Hippocampus isolation was carried out as described in Babu et al. [48]. Briefly, following animal decapitation, the head was recovered and the skull was opened with sterile scissors. Brain was carefully removed, quickly washed in ice-cold sterile saline solution and put on an ice-chilled plate. Immediately, it was cut along the longitudinal fissure to divide both

hemispheres. The diencephalon was removed with sterile microsurgical forceps and scissors and the exposed hippocampus was resected from the neocortex and immediately placed in liquid nitrogen.

RNA isolation and Reverse Transcriptase (RT) reaction

Total RNA was extracted from three pools of two hippocampi each. Hippocampus samples from control and vitamin A-deficient rats, were isolated at ZT2, ZT6, ZT10, ZT14, ZT18, and ZT22 (LD condition) and, CT2, CT6, CT10, CT14, CT18, and CT22 (DD condition). All RNA isolations were performed using the Trizol reagent (Invitrogen Co) as directed by the manufacturers. Gel electrophoresis and gel red staining confirmed the integrity of the samples. Quantification of RNA was based on spectrophotometric analysis at 260 nm. Three micrograms of total RNA were reverse-transcribed with 200 units of MMLV Reverse Transcriptase (Promega) using random hexamers in a 25- μ l reaction mixture and following manufacturer's instructions.

PCR amplification

Transcript levels of RAR α , RAR β , RXR β , BMAL1 and PER1 were determined by RT-PCR and normalized to 28S as endogenous control. Fragments coding for 28S, RAR α , RAR β , RXR β , BMAL1 and PER1 were amplified by PCR in 50 μ l of reaction solution containing 0.2 mM dNTPs, 1.5 mM MgCl₂, 1.25U of Taq polymerase, 50 pmol of each rat-specific oligonucleotide primer, and RT-generated cDNA (1/10 of RT reaction). The sequences of the specific primers are shown on Table 1. Samples were heated in a thermocycler (My Cycler, BioRad) to 94°C for 2 min, followed by 39 cycles of: (1) denaturation, 94°C for 1 min; (2) annealing, 59°C during 1 min; (3) extension, 72°C for 1 min. After 39 reaction cycles, the extension reaction was continued for another 10 min. In the case of RAR β , the thermocycling protocol was: 94°C for 4 min, followed by 35 cycles of: (1) denaturation, 94°C for 1 min; (2) annealing, 59°C during 1 min; (3) extension, 72°C for 1 min. After 35 reaction cycles, the extension reaction was continued for another 10 min. PCR products were then electrophoresed on 2% (w/v) agarose gel with 0.01% (w/v) gel red. The amplified fragments were visualized under ultraviolet transillumination and photographed using a Cannon PowerShot A75 3.2MP digital camera. The mean of gray value for each band was measured using the NIH ImageJ software (Image Processing and Analysis in Java from <http://rsb.info.nih.gov/ij/>) and the relative abundance of each band was normalized according to the arrhythmic housekeeping 28S gene, calculated as the ratio of the mean of gray value of each product to that of 28S.

Immunoblotting assays

Protein extracts were prepared from two pools of three hippocampi each, obtained from each group of rats at ZT2, ZT6, ZT10, ZT14, ZT18 and ZT22, for animals maintained under LD conditions, and at CT2, CT6, CT10, CT14, CT18, and CT22 for animals under DD conditions. Homogenates were prepared in buffer C (20 mM HEPES, pH 7.9, 0.42 M NaCl, 1.5 mM MgCl₂, 0.2 mM EDTA, 0.5 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride, 1 lg/ml leupeptin, 1 lg/ml of pepstatin, 1 mM sodium fluoride, 5 μ M sodium orthovanadate, and 25% glycerol). Aliquots containing 40 μ g of total protein were subjected to electrophoresis in 4–12% NuPage™ Bis-Tris gels (Invitrogen Life Technologies, Carlsbad, CA), and then transferred to Immobilon-PTM transfer membranes (Millipore, Bedford, MA). Immunoblot analyses were performed following the manufacturers' protocols for the detecting antibodies and as described in Fonzo et al [44]. Briefly, membranes were blocked in Blotto (5% nonfat dry milk 10 mM Tris-HCl, pH 8.0, and 150 mM NaCl) followed by 3-h incubation at RT with either rabbit anti-RAR α , RAR β and RXR β , goat anti-BMAL1, or rabbit anti-PER1 antibodies (Santa Cruz Biotechnology, Santa Cruz, CA) in Blotto containing 0.05% thimerosal. After incubation with primary antibody,

the membranes were washed in TBS (10 mM Tris-HCl, pH 8.0, and 150 mM NaCl) containing 0.05% Tween-20, before incubation with horseradish-peroxidase-conjugated donkey antigoat, or goat antirabbit, IgG (Santa Cruz Biotechnology) diluted 1:10,000 in Blotto for 1 h at room temperature. After washing, antibody/protein complexes on membranes were detected using Vectastain DAB Peroxidase Substrate kit from Vector Laboratories (Burlingame, CA) and following the manufacturers' indications. The mean of intensity of each band was measured using the NIH ImageJ software (Image Processing and Analysis in Java from <http://rsb.info.nih.gov/ij/>). RAR α , RAR β , RXR β , BMAL1 and PER1 protein levels were normalized against ACTIN (endogenous control).

Scanning of RAR α , RAR β , RXR α , PER1 y BMAL1 upstream regions for putative E-box, RARE and RXRE sites

To identify putative retinoic acid-responsive (RARE, AGGTCANNNNNAGGTCA, and RXRE, AGGTCANAGGTCA) as well as clock-responsive (perfect E-box: CACGTG, or E-box like: CANNTG) DNA consensus regulatory sites, RARs, RXRs and clock genes up to 1500 bp upstream of the translation start codon, were scanned for significant matches using the MatInspector software from Genomatix (<http://www.genomatix.de>; [49]).

Statistical Analysis

Time point data were expressed as means $\pm$ standard errors of the mean (SE) and pertinent curves were drawn. Time series were computed first by one-way ANOVA followed by Tukey post-hoc test for specific comparisons; a $p < 0.05$ was considered to be significant. When amplitude or phase was required, a fitting technique was applied. Data were fitted by the following function: $c + a \cos[2\pi(t - \phi)/24]$, where c is the mesor, a is the amplitude of the cosine wave, t is time in hours, and ϕ is the phase in hours from ZT 0 in the imposed LD cycle and CT 0 in DD condition. The fitting was performed using Nonlinear Regression from GraphPad Prism 3.0 software (CA, USA). The routine also estimates the standard error of the fit parameters. The standard error arises from scatter in the data and from deviations of the data from cosine form. Note that the frequency was taken as the 1 cycle per 24 h of the light regime. The standard error of a phase difference was calculated as the square root of the sum of the squared standard errors of the differenced phases. Additionally to the significance of conventional statistics, chronobiologic statistics were used for validating temporal changes as rhythms. Thus, each series was analyzed by single and population-mean Cosinor method (Halberg Chronobiology Center, <http://www.msi.umn.edu/~halberg/>) at a trial period of 24 h [50, 51, 52]. The cosinor method is an inferential statistical method that fits one (or several) cosine curve(s) by least squares to the data, yielding estimates for the mesor (a rhythm-adjusted mean), and for the amplitude and acrophase (measures of the extent and timing of predictable change within a cycle). Based on the residual sum of squares, a P value was derived for the zero-amplitude (no-rhythm) test and for the computation of confidence intervals of 95% for the parameters [52]. A $p \leq 0.05$ or less was taken as indicative of the presence of a rhythm with the 24h (anticipated) period. Student's t -test was used for comparison of mesor, amplitude or acrophase between LD vs DD or CO vs VAD groups, with $p < 0.05$ for significant differences.

RESULTS

Daily profiles of RAR α , RAR β and RXR β mRNA and protein levels in rat hippocampus

In order to assess whether RAR α , RAR β and RXR β mRNA and protein expression varies throughout a 24h period in the rat hippocampus, samples from animals maintained under LD conditions were analysed for transcripts and protein levels over a period of 24 hours. Interestingly, both mRNA and protein levels of retinoic acid receptors vary significantly throughout the day in the hippocampus of control rats (Figures 1A-C, $p < 0.01$ and Table 2;

2A-C, $p < 0.05$ and Table 3). The $RAR\alpha$, $RAR\beta$ y $RXR\beta$ transcript levels peak around the middle of the night with acrophases at ZT 18:42±01:08, 15:30±00:31, and 17:32±00:32, respectively, while the highest level of proteins was observed at ZT 08:20±01:12, 20:25±00:14 and 20:31±01:39, respectively.

Circadian Patterns of transcripts and protein levels of $RAR\alpha$, $RAR\beta$ y $RXR\beta$ in the rat Hippocampus

Once we observed retinoic acid receptors display daily patterns of transcripts and protein levels, we wondered whether these expression patterns could be synchronized endogenously. For that, samples were obtained from control rats maintained under DD conditions. Tissue extracts were prepared as described in the Materials and Methods section, and subjected to RT-PCR and immunoblotting. The results revealed that mRNA expression as well as protein levels, follow a robust circadian rhythm in the hippocampus of control rats. The $RAR\alpha$, $RAR\beta$ y $RXR\beta$ transcript levels peaks occurred in the first half of the subjective night in the rat hippocampus with acrophases at CT 18:50±00:24, 19:13±00:06, and 18:00±00:34, respectively (Figures 3A-C, $p < 0.01$ and Table 2). We found, that the highest protein levels occur at CT 16:25±00:19, 02:12±00:02 and 15:30±00:50 respectively (Figures 4A-C, $p < 0.01$ and Table 3).

Circadian rhythms of $RAR\alpha$, $RAR\beta$ y $RXR\beta$ expression mRNA and protein in the hippocampus of vitamin A-deficient rats

Circadian oscillating expression of acid receptors was differentially affected by the nutritional vitamin A deficiency. On one hand, the VAD phase shifted the circadian pattern of $RAR\alpha$, $RAR\beta$ and $RXR\beta$ mRNA (CT 18:50±00:24 vs 16:29±00:10, $p < 0.01$; 19:13±00:06 vs 7:00±00:00 $p < 0.05$ and 18:00±00:34 vs 15:26±00:50 $p < 0.05$ respectively; Figures 3A-C, Table 4) and protein levels (CT 16:25±00:20 vs 13:55±00:02, $p < 0.01$, 02:12±00:02 vs 15:51±00:27, $p < 0.01$; and 15:30±00:50 vs 10:10±00:54, $p < 0.01$; respectively; Figures 4A-C, Table 5). Additionally, VAD attenuated the rhythms of $RAR\alpha$ mRNA and $RAR\beta$ protein expressions (amplitude: 0.26±0.05 vs 0.05±0.02, $p < 0.01$; and 0.21±0.01 vs 0.07±0.03, $p < 0.01$, respectively; Figures 4A-C, Table 5).

Putative E-box sites on $RAR\alpha$, $RAR\beta$ and $RXR\beta$ genes upstream region

Once we had knowledge retinoic acid receptors display circadian expression patterns in the rat hippocampus, and that these are modified by the vitamin A deficiency, we scanned regulatory regions of $RAR\alpha$, $RAR\beta$ y $RXR\beta$ genes in order to look for clock-responsive sites. The analysis of 1200 bp upstream of the translation start codon in the Genomatix database revealed one perfect E-box and four E-box-like sites in the $RAR\alpha$ regulatory region while four E-box-like elements were found in the $RAR\beta$ and three in the $RXR\beta$ promoter. Additionally, one and four retinoic acid-related orphan receptor a ($ROR\alpha$) responsive elements (ROREs) were found on the $RAR\alpha$ and $RAR\beta$ promoters, respectively (Figure 5).

Temporal expression of mRNA and protein BMAL1 and PER1 in the hippocampus of vitamin A-deficient rats

Finding clock-responsive sites on the gene regulatory regions of retinoic acid receptors and the observation that their circadian expression pattern is modified in the hippocampus of vitamin A-deficient rats, led us to test whether and to which extent VAD could affect the circadian expression of key clock factors. Thus, we analyzed the oscillating expression of BMAL1 and PER1 mRNA and protein levels, in the hippocampus of control and vitamin A-deficient rats maintained under DD conditions. We found BMAL1 and PER1 mRNA expression display circadian profiles in the rat hippocampus (Figures 6A-B, $p < 0.05$) with

their acrophases occurring at CT 15:30±01:15 and 17:55±00:21, respectively (Figure 6A and B and Table 6). Circadian variation of protein levels were also observed in that brain area (Figures 7A-B and $p<0.01$) with BMAL1 peaking at CT 04:31±01:12 and PER1 at CT 22:44±00:40 (Table 7). Interestingly, VAD phase shifted the circadian pattern of BMAL1 mRNA and protein (acrophases shifted from CT 15:30±01:15 to 02:33±00:33 and from CT 04:31±01:12 to 12:40±00:33, respectively). However, the nutritional deficiency abolished PER1 mRNA and protein rhythms (Figure 6A-B and 7A-B).

Putative RARE and RXRE sites on BMAL1 and PER1 genes upstream region

Scanning of 1500 pb upstream of the translation start codon in BMAL1 and PER1 genes in the Genomatix database, is showed in Figure 8. The analysis revealed three RXREs and one RARE site on the BMAL1 while two RXREs and one RARE site were found on the PER1 gene regulatory regions.

DISCUSSION

Vitamin A may modulate learning and memory-underlying LTP through activation of its nuclear receptors, RARs and RXRs [37]. Here we show that expression of RAR α , RAR β and RXR β is rhythmic and circadian in the hippocampus. First, we investigated whether retinoic acid receptors, mRNA and protein levels, showed a day-night LD oscillation in the rat hippocampus. Interestingly, we observed RAR α , RAR β and RXR β , mRNA and protein levels, vary throughout a 24h period in the rat hippocampus, with transcripts peaking around the middle of the night (Figure 1A-C and Table 2) and preceding protein levels acrophases in 14, 5, and 3 hours, respectively (Figures 1 and 2 and Tables 2 and 3). Rhythms of RAR and RXR receptors have also been observed by us and others in different species and tissues [46,53]. These observations led us to question whether these rhythms were endogenously-driven and whether retinoic acid receptors expression moved in time to the cellular clock in the hippocampus.

In order to that, we continued analyzing RAR α , RAR β and RXR β mRNA and protein levels in the hippocampus of animals maintained under free running (DD) conditions. In fact, and for the first time in our knowledge, we observed transcript and protein levels continued oscillating in this brain area under constant darkness (Figures 3 and 4); this and the cosinor validation would allow us to name them circadian rhythms. As expected, circadian patterns of RAR α , RAR β and RXR β mRNA were in phase with their daily profiles (Figures 1 and 3, Table 2) in the hippocampus of control rats. However, only the RXR β protein levels oscillate with the same pattern in LD and DD conditions (Figures 2C and 4C, Table 3). The discrepancies between circadian (DD) and daily (LD) patterns of RAR α and RAR β protein levels might be due, at least in part, to differences in their posttranscriptional regulation under different lighting conditions; although masking effects of light on endogenous RAR α and RAR β rhythms might also be occurring.

Circadian rhythmicity of retinoic acid receptors expression may be either a direct or an indirect output of the hippocampal molecular clock. It is known that the cellular molecular clock drives the oscillating expression of controlled genes through the binding of the BMAL1:CLOCK heterodimer to E-box elements in their promoters [54, 55]. We found one perfect E-box and four E-box-like sites in the RAR α regulatory region while four E-box-like elements were found in the RAR β and three in the RXR β promoter (Figure 5), however, the peaks of retinoic acid receptors mRNA appear around 14 h later than BMAL1 protein acrophase (Figures 3 and 7, Tables 2 and 7). Even though the non-canonical E-box-like sequences have also been reported to be necessary for circadian oscillation, preferentially, they should be in tandem spaced by 6-8 nucleotides between them to be functional [56]. Our analysis of clock's transcription factor binding sites revealed that only in the RAR α

promoter a perfect E-box and an E-box-like site are separated by only 9 bp while in the RAR β and RXR β regulatory regions E-box-like sites are more spaced one from other. This would lead us to think that RARs and RXR β circadian rhythmicity would probably be regulated by the clock in an indirect way. For example, since we and others [57] (Matsui et al) have found ROR-responsive sites (ROREs) on the RARs genes regulatory regions (Figure 5), their oscillating expression patterns could be a result of a circadian ROR α /REVERB-mediated regulation [39, 58, 59]. Indeed, we recently showed that BMAL1 target, transcriptional activator ROR α peaks at ZT14:21, in antiphase with repressor REVERB protein [60]. ROR α protein acrophase precedes in around 4 hours RARs and RXR β transcripts peaks showed in this work suggesting ROR α might be activating retinoic acid receptors transcription in the hippocampus. Reciprocally, it has been shown that ROR α -mediated transcription is activated synergistically by RAR in Pukinje cerebellar cells [57], evidencing a functional interaction between ROR α and RAR transcription factors.

Interestingly, we observed that circadian rhythmicity of retinoic acid receptors is modified in the hippocampus of vitamin A-deficient rats. The VAD phase-shifted, and also attenuated, circadian rhythmicity of RAR α and RAR β mRNA and proteins; however, it only phase shifted RXR β circadian expression, in the rat hippocampus (Figures 3 and 4 A-C, Tables 4 y 5). It is well known that RARs and RXRs receptors function as transcription factors which regulate the expression of target genes by direct binding to retinoic acid response elements, RAREs for RAR:RXR heterodimers and RXREs for RXR:RXR homodimers, in their target genes promoter [31,32,33]. The sequence analysis revealed the presence of one RARE and three RXREs on the BMAL1 regulatory region and one RARE and two RXRE sites on the PER1 promoter (Figure 6). On the other hand, we observed that RAR α and RXR β protein peaks are in phase with the circadian BMAL1 and PER1 mRNA expression. These observations would suggest that vitamin A might have a role in the regulation of clock genes expression. For example, moderate phase shifts of peripheral clocks in the heart and aorta have been observed after injection of retinoic acid [34]. Additionally, looking for potential factors which could mediate entrainment of circadian rhythms in cultured fibroblasts expressing Per2-luciferase, Nakahata et al. [61] found that 3 out of 12 active targets were *all-trans*-retinoic acid, *9-cis* retinoic acid, and *13-cis* retinoic acid. Interestingly, in our study, the lack of vitamin A in the diet affected not only its nuclear receptors expression but also the circadian rhythmicity of clock genes expression in the hippocampus. Thus, and similar to what we previously observed under LD conditions in this brain area as well as in liver [44, 46], VAD phase shifted BMAL1 and abolished PER1 circadian expression at both, mRNA and protein levels (Figures 7 and 8 and Tables 6 and 7). This certainly affects the endogenous clock activity in the hippocampus and, consequently, the temporal organization of some target functions, such as could be the daily performance of memory and learning. In fact, several studies suggest an association between cellular molecular clock disruptions and impaired cognitive functions [62, 63, 64, 65].

VAD is a serious concern and has a clinical and socio-economical significance worldwide. We would expect that the emerging data from this study will also highlight retinoid signaling pathways as potential novel therapeutic targets for cognitive deficits and/or clock-related disorders.

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References

1. Cailotto C, van Heijningen C, van der Vliet J, van der Plasse G, Habold C, Kalsbeek A, Pévet P, Buijs RM. Daily rhythms in metabolic liver enzymes and plasma glucose require a balance in the autonomic output to the liver. *Endocrinology*. 2007; 149:1914–25. [PubMed: 18162516]
2. Lamia KA, Storch KF, Weitz CJ. Physiological significance of a peripheral tissue circadian clock. *Proc Natl Acad Sci USA*. 2008; 105:15172–77. [PubMed: 18779586]
3. Froy O. The circadian clock and metabolism. *Clin Sci (Lond)*. 2011; 120:65–72. [PubMed: 20929440]
4. Chaudhury D, Wang LM, Colwell CS. Circadian Regulation of Hippocampal Long-Term Potentiation. *J Biol Rhythm*. 2005; 20:225–236.
5. Golombek DA, Rosenstein RE. Physiology of Circadian Entrainment. *Physiol Rev*. 2010; 90:1063–1102. [PubMed: 20664079]
6. Aschoff J. Circadian rhythms in man. *Science*. 1965; 148:1427–32. [PubMed: 14294139]
7. Aschoff J, Wever R. Human circadian rhythms: a multioscillatory system. *Federation Proc*. 1976; 35:232–36.
8. Sangoram AM, Saez L, Antoch MP, Gekakis N, Staknis D, Whiteley A, Fruechte EM, Vitaterna MH, Shimomura K, King DP, Young MW, Weitz CJ, Takahashi JS. Mammalian circadian autoregulatory loop: a timeless ortholog and mPer1 interact and negatively regulate CLOCK-BMAL1-induced transcription. *Neuron*. 1998; 21:1101–13. [PubMed: 9856465]
9. Reppert SM, Weaver DR. Coordination of circadian timing in mammals. *Nature*. 2002; 418:935–41. [PubMed: 12198538]
10. Panda S, Hogenesch JB, Kay SA. Circadian light input in plants, flies and mammals. *Novartis Found Symp*. 2003; 253:73–82. [PubMed: 14712915]
11. Schibler U, Sassone-Corsi P. A web of circadian pacemakers. *Cell*. 2002; 111:919–22. [PubMed: 12507418]
12. Hirota T, Fukada Y. Resetting mechanism of central and peripheral circadian clocks in mammals. *Zoolog Sci*. 2004; 21:359–68. [PubMed: 15118222]
13. Panda S, Hogenesch JB. It's all in the timing: many clocks, many outputs. *J Biol Rhythms*. 2004; 19:374–387. [PubMed: 15534318]
14. Gekakis N, Staknis D, Nguyen HB, Davis FC, Wilsbacher LD, King DP, Takahashi JS, Weitz CJ. Role of the CLOCK protein in the mammalian circadian mechanism. *Science*. 1998; 280:1564–69. [PubMed: 9616112]
15. Hogenesch JB, Gu YZ, Jain S, Bradfield CA. The basic-helix-loop-helix-PAS orphan MOP3 forms transcriptionally active complexes with circadian and hypoxia factors. *Proc Natl Acad Sci U S A*. 1998; 95:5474–79. [PubMed: 9576906]
16. King DP, Takahashi JS. Molecular genetics of circadian rhythms in mammals. *Annu Rev Neurosci*. 2000; 23:713–42. [PubMed: 10845079]
17. Lee C, Weaver DR, Reppert SM. Direct Association between Mouse PERIOD and CKIε Is Critical for a Functioning Circadian Clock. *Mol Cell Biol*. 2004; 24:584–94. [PubMed: 14701732]
18. Vanselow K, Kramer A. Role of phosphorylation in the mammalian circadian clock. *Cold Spring Harb Symp Quant Biol*. 2007; 72:167–76. [PubMed: 18419274]
19. Sato TK, Yamada RG, Ukai H, Baggs JE, Miraglia LJ, Kobayashi TJ, Welsh DK, Kay SA, Ueda HR, Hogenesch JB. Feedback repression is required for mammalian circadian clock function. *Nat Genet*. 2006; 38:312–19. [PubMed: 16474406]
20. Preitner N, Damiola F, Lopez-Molina L, Zakany J, Duboule D, Albrecht U, Schibler U. The orphan nuclear receptor REV-ERBa controls circadian transcription within the positive limb of the mammalian circadian oscillator. *Cell*. 2002; 110:251–60. [PubMed: 12150932]
21. Sato TK, Panda S, Miraglia LJ, Reyes TM, Rudic RD, McNamara P, Naik KA, FitzGerald GA, Kay SA, Hogenesch JB. A functional genomics strategy reveals Rora as a component of the mammalian circadian clock. *Neuron*. 2004; 43:527–37. [PubMed: 15312651]
22. Akashi M, Takumi T. The orphan nuclear receptor RORα regulates circadian transcription of the mammalian core-clock Bmal1. *Nat Struct Mol Biol*. 2005; 12:441–48. [PubMed: 15821743]

23. Guillaumond F, Dardente H, Giguere V, Cermakian N. Differential control of Bmal1 circadian transcription by REV-ERB and ROR nuclear receptors. *J Biol Rhythms*. 2005; 20:391–403. [PubMed: 16267379]
24. Johnson RF, Moore RY, Morin LP. Loss of entrainment and anatomical plasticity after lesions of the hamster retinohypothalamic tract. *Brain Res*. 1998; 460:297–313. [PubMed: 2465060]
25. Gooley JJ, Lu J, Chou TC, Scammell TE, Saper CB. Melanopsin in cells of origin of the retinohypothalamic tract. *Nat Neurosci*. 2001; 4:1165. [PubMed: 11713469]
26. Palmer JD. The clocks controlling the tide-associated rhythms of intertidal animals. *Bioessays*. 2000; 22:32–37. [PubMed: 10649288]
27. Feillet CA, Albrecht U, Challet E. “Feeding time” for the brain: a matter of clocks. *J Physiol*. 2006; 100:252–60.
28. Mendoza J. Circadian clocks: setting time by food. *J Neuroendocrinol*. 2007; 19:127–37. [PubMed: 17214875]
29. Damiola F, Le Minh N, Preitner N, Kornmann B, Fleury-Olela F, Schibler U. Restricted feeding uncouples circadian oscillators in peripheral tissues from the central pacemaker in the suprachiasmatic nucleus. *Genes Dev*. 2000; 14:2950–2961. [PubMed: 11114885]
30. Cassone VM, Stephan FK. Central and peripheral regulation of feeding and nutrition by the mammalian circadian clock: implications for nutrition during manned space flight. *Nutrition*. 2002; 18:814–819. [PubMed: 12361772]
31. Heyman RA, Mangelsdorf DJ, Dyck JA, Stein RB, Eichele G, Evans RM, Thaller C. 9-Cis retinoic acid is a high affinity ligand for the retinoid X receptor. *Cell*. 1992; 68:397–406. [PubMed: 1310260]
32. Mangelsdorf DJ, Thummel C, Beato M, Herrlich P, Schutz G, Umesono K, Blumberg B, Kastner P, Mark M, Chambon P, Evans R. The nuclear receptor superfamily: the second decade. *Cell*. 1995; 83:835–39. [PubMed: 8521507]
33. Soprano DR, Qin P, Soprano KJ. Retinoic acid receptors and cancers. *Annu Rev Nutr*. 2004; 24:201–21. [PubMed: 15189119]
34. McNamara P, Seo SP, Rudic RD, Sehgal A, Chakravarti D, FitzGerald GA. Regulation of CLOCK and MOP4 by nuclear hormone receptors in the vasculature: a humoral mechanism to reset a peripheral clock. *Cell*. 2001; 105:877–89. [PubMed: 11439184]
35. Misner DL, Jacobs S, Shimizu Y, de Urquiza AM, Solomin L, Perlmann T, De Luca LM, Stevens CF, Evans RM. Vitamin A deprivation results in reversible loss of hippocampal long-term synaptic plasticity. *Proc Natl Acad Sci U S A*. 2001; 98:11714–19. [PubMed: 11553775]
36. Cocco S, Diaz G, Stancampiano R, Diana A, Carta M, Curreli R, Sarais L, Fadda F. Vitamin A deficiency produces spatial learning and memory impairment in rats. *Neuroscience*. 2002; 115:475–82. [PubMed: 12421614]
37. McCaffery P, Zhang J, Crandall JE. Retinoic acid signaling and function in the adult hippocampus. *J Neurobiol*. 2006; 66:780–91. [PubMed: 16688774]
38. Chiang M-Y, Misner D, Kempermann G, Schikorski T, Giguere V, Sucov HM, Gage FH, Stevens CF, Evans RM. An Essential Role for Retinoid Receptors RAR β and RXR γ in Long-Term Potentiation and Depression. *Neuron*. 1998; 21:1353–61. [PubMed: 9883728]
39. Etchamendy N, Enderlin V, Marighetto A, Pallet V, Higuieret P, Jaffard R. Vitamin A deficiency and relational memory deficit in adult mice: Relationships with changes in brain retinoid signalling. *Behav Brain Res*. 2003; 145:37–49. [PubMed: 14529804]
40. Winocur G, Hasher L. Aging and time-of-day effects on cognition in rats. *Behav Neurosci*. 1999; 113:991–97. [PubMed: 10571481]
41. Winocur G, Hasher L. Circadian rhythms and memory in aged humans and animals. In: Squire, LR.; Schacter, DL., editors. *Neuropsychology of memory*. 3. New York: Guilford Press; 2002. p. 273-85.
42. Winocur G, Hasher L. Age and time-of-day effects on learning and memory in a non-matching-to-sample test. *Neurobiol Aging*. 2004; 25:1107–15. [PubMed: 15212835]
43. Valentinuzzi VS, Neto SP, Carneiro BT, Santana KS, Araújo JF, Ralph MR. Memory for time of training modulates performance on a place conditioning task in marmosets. *Neurobiol Learn Mem*. 2008; 89:604–7. [PubMed: 17904878]

44. Fonzo LS, Golini R, Delgado SM, Bonomi MR, Rezza IG, Gimenez MS, Anzulovich AC. Temporal patterns of lipoperoxidation and antioxidant enzymes are modified in the hippocampus of vitamin A-deficient rats. *Hippocampus*. 2009; 19:869–80. [PubMed: 19308957]
45. Vega VA, Anzulovich AC, Varas SM, Bonomi MR, Giménez MS, Oliveros LB. Effect of nutritional vitamin A deficiency on lipid metabolism in the rat heart: Its relation to PPAR gene expression. *Nutrition*. 2009; 25:828–838. [PubMed: 19342198]
46. Ponce IT, Rezza IG, Delgado SM, Navigatore LS, Bonomi MR, Golini R, Gimenez MS, Anzulovich AC. Daily oscillation of glutathione redox cycle is dampened in the nutritional vitamin A deficiency. *Biol Rhythm Res*. 2011 Available on line: 01 Aug 2011.
47. Reeves PG, Nielsen FH, Fahey GC Jr. AIN- purified diets for laboratory rodents: Final report of the American Institute of Nutrition ad hoc writing committee on the reformulation of the AIN-76A rodent diet. *J Nutr*. 1993; 123:1939–51. [PubMed: 8229312]
48. Babu H, Claasen JH, Kannan S, Rünker AE, Palmer T, Kempermann G. A protocol for isolation and enriched monolayer cultivation of neural precursor cells from mouse dentate gyrus. *Front Neurosci*. 2011; 5:89. [PubMed: 21811434]
49. Quandt K, Frech KK, Karas H, Wingender E, Werner T. MatInd and MatInspector: new fast and versatile tools for detection of consensus matches in nucleotide sequence data. *Nucleic Acids Res*. 1995; 23:4878–84. [PubMed: 8532532]
50. Halberg F. Chronobiology. *Ann Rev Physiol*. 1969; 31:675–725. [PubMed: 4885778]
51. Bingham C, Arbogast B, Cornelissen-Guillaume G, Lee JK, Halberg F. Inferential statistical methods for estimating and comparing Cosinor parameters. *Chronobiologia*. 1982; 9:397–439. [PubMed: 7168995]
52. Thaela MJ, Jensen MS, Cornélissen G, Halberg F, Nöddegaard F, Jakobsen K, Pierzynowski SG. Circadian and ultradian variation in pancreatic secretion of meal-fed pigs after weaning. *J Anim Sci*. 1998; 76:1131–1139. [PubMed: 9581937]
53. Yang X, Downes M, Yu RT, Bookout AL, He W, Straume M, Mangelsdorf DJ, Evans RM. Nuclear receptor expression links the circadian clock to metabolism. *Cell*. 2006; 126:801–10. [PubMed: 16923398]
54. Pando MP, Sassone-Corsi P. Signaling to the mammalian circadian clocks: in pursuit of the primary mammalian circadian photoreceptor. *Science STKE*. 2001; 6:re16.
55. Reppert SM, Weaver DR. Molecular analysis of mammalian circadian rhythms. *Annu Rev Physiol*. 2001; 63:647–76. [PubMed: 11181971]
56. Nakahata Y, Yoshida M, Takano A, Soma H, Yamamoto T, Yasuda A, Nakatsu T, Takumi T. A direct repeat of E-box-like elements is required for cell-autonomous circadian rhythm of clock genes. *BMC Mol Biol*. 2008; 4(9):1. [PubMed: 18177499]
57. Matsui T. Transcriptional regulation of a Purkinje cell-specific gene through a functional interaction between ROR alpha and RAR. *Genes cells*. 1997; 2:263–72. [PubMed: 9224660]
58. Ueda HR, Hayashi S, Chen WB, Sano M, Machida M, Shigeyoshi Y, Iino M, Hashimoto S. System level identification of transcriptional circuits underlying mammalian circadian clocks. *Nat Genet*. 2005; 37:187–192. [PubMed: 15665827]
59. Crumbley C, Wang Y, Kojetin DJ, Burris TP. Characterization of the core mammalian clock component, NPAS2, as a REV-ERBalpha/RORalpha target gene. *J Biol Chem*. 2010; 285:35386–92. [PubMed: 20817722]
60. Golini RS, Delgado SM, Navigatore-Fonzo LS, Ponce IT, Lacoste MG, Anzulovich AC. Daily patterns of clock and cognition-related factors are modified in the hippocampus of vitamin a-deficient rats. *Hippocampus*. 2011 Accepted for publication.
61. Nakahata Y, Akashi M, Trecka D, Yasuda A, Takumi T. The in vitro real-time oscillation monitoring system identifies potential entrainment factors for circadian clocks. *BMC Mol Biol*. 2006; 7:5. [PubMed: 16483373]
62. Garcia JA, Zhang D, Estill SJ, Michnoff C, Rutter J, Reick M, Scott K, Diaz-Arrastia R, McKnight SL. Impaired cued and contextual memory in NPAS2-deficient mice. *Science*. 2000; 288:2226–30. [PubMed: 10864874]
63. Abarca C, Albrecht U, Spanagel R. Cocaine sensitization and reward are under the influence of circadian genes and rhythm. *Proc Natl Acad Sci U S A*. 2002; 99:9026–30. [PubMed: 12084940]

64. Hampp G, Albrecht U. The circadian clock and mood-related behavior. *Commun Integr Biol.* 2008; 1:1–3. [PubMed: 19704445]
65. Jilg A, Lesny S, Peruzki N, Schwegler H, Selbach O, Dehghani F, Stehle JH. Temporal dynamics of mouse hippocampal clock gene expression support memory processing. *Hippocampus.* 2010; 20:377–88. [PubMed: 19437502]

Abbreviations

9cisRA	9-cis-retinoic acid
ANOVA	analysis of variance
ATRA	all-trans-retinoic acid
LTP	long term potentiation
RAR	retinoic acid receptor
RARE	retinoic acid responsive element
RORE	retinoid-related orphan receptor responsive element
RXR	retinoid X receptor
RXRE	retinoid X responsive element
SCN	suprachiasmatic nucleus
ZT	zeitgeber time
CT	circadian time

Figure 1. Daily rhythms of RARα (A), RARβ (B) and RXRβ (C) mRNA expression in the rat hippocampus. Horizontal bars represent the distribution of light (open) and dark (closed) phases of a 24 h (ZT0-ZT24) photoperiod. Cosine fitting curves represent normalized mRNA levels versus ZT. Each value on the curve represents the mean $\pm$ SE of three pools of two hippocampus samples each at a given ZT (with ZT= 0 when light is on). Statistical analysis was performed using one-way ANOVA followed by Tukey test with * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$ when indicated means were compared to the corresponding maximal value in each group. Top of the figures, $p = 0.05$ indicates detection of a rhythm, $p = 0$ indicates $p < 0.0005$ from analysis by the cosinor method. D) Representative patterns of PCR products at different ZTs throughout a day-night cycle.

Figure 2.

Day-night cycles of RARα (A), RARβ (B), and RXRβ (C) protein levels in the rat hippocampus. Cosine fitting curves for rhythmic normalized RARα, RARβ, and RXRβ protein levels obtained from the densitometric quantification of the Immunoblots representative data. Each point represents the mean $\pm$ SE of two pools of two hippocampus samples each at a given ZT (with ZT= 0 when light is on). Horizontal bars represent the distribution of light (open) and dark (closed) phases of the 24-h photoperiod. Statistical analysis was performed using one-way ANOVA followed by Tukey test with * $P < 0.05$ when indicated means were compared to the corresponding maximal value in each group. Top of the figures, $p \leq 0.05$ indicates detection of a rhythm, $p = 0$ indicates $p < 0.0005$ from analysis by the cosinor method. D) Representative Immunoblots at different ZTs throughout a day-night cycle.

Figure 3. Circadian rhythms of RARα, RARβ, and RXRβ mRNA levels in the hippocampus of CO and VAD rats. Cosine fitting curves for normalized RARα (A), RARβ (B), and RXRβ (C) transcripts levels throughout a day. Horizontal bars represent the distribution of dark-dark (DD) phases of a 24 h photoperiod (CT0-CT24). Each point on the curve represents the mean $\pm$ SE of three pools of two hippocampus samples each at a given CT. Statistical analysis was performed using one-way ANOVA followed by Tukey test with $*P < 0.05$, $**P < 0.01$ and $***P < 0.001$ when indicated means were compared to the corresponding maximal value in each group. Top of the figures, $p = 0.05$ indicates detection of a rhythm, $p = 0$ indicates $p < 0.0005$ from analysis by the cosinor method. D) Representative patterns of PCR products at different CTs in a 24 h cycle.

Figure 4.

Circadian profiles of RARα (A), RARβ (B), and RXRβ (C) protein levels in the hippocampus of CO and VAD rats. Cosine fitting curves for rhythmic normalized RARα, RARβ, and RXRβ protein levels were obtained from the densitometric quantification of the Immunoblots representative data. Each point in the curve represents the mean $\pm$ SE of two pools of two hippocampus samples each at a given CT. Horizontal bars represent the distribution of dark-dark (DD) phases of a 24-h photoperiod. Statistical analysis was performed using one-way ANOVA followed by Tukey test with $*P<0.05$, $**P<0.01$ and $***P<0.001$ when indicated means were compared to the corresponding maximal value in each group. Top of the figures, $p=0.05$ indicates detection of a rhythm, $p=0$ indicates $p<0.0005$ from analysis by the cosinor method. D) Representative Immunoblots at different CTs in a 24 h cycle.

Figure 5.

Schematic representation of RARE, RXRE, and E-box sites on the 5' regulatory region of *RARα*, *RARβ* and *RXRβ* genes. Arrows indicate the first translation codon, gray boxes represent exons, black circles are RARE sites, dashed circles RXREs, white circles represents RORE sites and gray ovals are E-box-like (CATATG, CATGTG, or CACTTG) sites. Negative (-) numbers indicate regulatory sites positions relative to the start of translation (+1).

Figure 6.

Circadian patterns of BMAL1 and PER1 mRNA levels in the hippocampus of CO and VAD rats. Cosine fitting curves for normalized BMAL1 (A) and PER1 (B) mRNA levels throughout a day. Each point in the curve represents the mean $\pm$ SE of two pools of two hippocampus samples each at a given CT. Horizontal bars represent the distribution of dark-dark (DD) phases of the 24-h photoperiod. Statistical analysis was performed using one-way ANOVA followed by Tukey test with $*P < 0.05$ and $**P < 0.01$ when indicated means were compared to the corresponding maximal value in each group. Top of the figures, $p = 0.05$ indicates detection of a rhythm, $p = 0$ indicates $p < 0.0005$ from analysis by the cosinor method. C) Representative patterns of PCR products at different CTs in a 24 h cycle.

Figure 7. Circadian profiles of BMAL1(A) and PER1(B) protein levels in the hippocampus of CO and VAD rats. Cosine fitting curves for rhythmic normalized BMAL1 and PER1 protein levels were obtained from the densitometric quantification of the Immunoblots representative data. Each point in the curve represents the mean $\pm$ SE of two pools of two hippocampus samples each at a given CT. Horizontal bars represent the distribution of dark-dark (DD) phases of the 24-h photoperiod. Statistical analysis was performed using one-way ANOVA followed by Tukey test with $*P < 0.05$, $**P < 0.01$ and $***P < 0.001$ when indicated means were compared to the corresponding maximal value in each group. Top of the figures, $p = 0.05$ indicates detection of a rhythm, $p = 0$ indicates $p < 0.0005$ from analysis by the cosinor method. C) Representative Immunoblots at different CTs in a 24 h cycle.

Figure 8. Schematic representation of RARE and RXRE sites on the 5' regulatory region of BMAL1 and PER1 genes. Arrows indicate the first translation codon, gray boxes represent exons, dashed circles are RXRE sites and black circles are RAREs. Negative (-) numbers indicate regulatory sites positions relative to the start of translation (+1).

Table 1

Primer pairs used for RT-PCR*

Gene name	GenBank Accession No	Forward primer 5'- 3'	Reverse primer 5'- 3'	Fragm. size
BMAL1	AB012600	GGGAAATACGGGTGAAGTCTATGG	ATGCCTGGAAGAGTGGGATGAGTC	460 bp
PER1	AB092976	ATTCCGCCTAACCCCATATGT	GTGTGCCGTGTGGTGAAGAT	150 bp
RARα	NM_031528	CGCCTGTGAGGGCTGTAAG	ATGCCCACTTCGAAGCATTT	150 bp
RARβ	XM_223843	GCCTGCAGAAGTGCTTTGAAG	GGCAGAGTGAGGAAAGGTTT	180 bp
RXRβ	NM_206849	CGAAGCTCAGGCAAGCACTA	TCCTGTACCGCCTCCCTTTT	200 bp

Table 2
Rhythm parameters of retinoic acid receptors mRNA oscillating levels in the rat hippocampus

Rhythm Parameters	Mesor (mean ± SE)		Amplitude (mean ± SE)		Acrophase (mean ± SE)	
	LD	DD	p	LD	DD	p
RARα	0.97±0.0	0.81±0.0	<0.01	0.12±0.0	0.26±0.0	ns
RARβ	1.13±0.0	0.60±0.0	<0.01	0.11±0.0	0.03±0.0	<0.01
RXRβ	0.82±0.0	0.41±0.0	<0.01	0.18±0.0	0.11±0.0	ns

p-levels were obtained for the corresponding LD vs. DD comparisons.

ns: not significant.

Table 3
Rhythm parameters of retinoic acid receptors protein oscillating levels in the rat hippocampus

Rhythm Parameters	Mesor (mean ± SE)			Amplitude (mean ± SE)			Acrophase (mean ± SE)		
	LD	DD	p	LD	DD	p	LD	DD	p
RARα	1.09±0.00	1.15±0.00	ns	0.23±0.01	0.37±0.01	ns	08:20±01:12	16:25±00:19	<0.01
RARβ	1.05±0.00	1.84±0.00	<0.01	0.56±0.01	0.21±0.00	<0.05	20:25±00:14	02:12±00:02	<0.01
RXRβ	1.28±0.00	1.71±0.00	ns	0.06±0.00	0.40±0.2	ns	20:31±01:39	15:30±00:50	ns

p-levels were obtained for the corresponding LD vs. DD comparisons.

ns: not significant.

Rhythm parameters of circadian retinoic acid receptors mRNA oscillating levels in the hippocampus of CO and VAD rats.

Table 4

Rhythm Parameters	Mesor (mean ± SE)		Amplitude (mean ± SE)		Acrophase (mean ± SE)	
	CO	VAD	p	CO	VAD	p
RARα	0.81 ± 0.0	0.28 ± 0.0	<0.01	0.26 ± 0.05	0.05 ± 0.02	<0.01
RARβ	0.60 ± 0.0	0.55 ± 0.0	<0.05	0.03 ± 0.0	0.03 ± 0.0	ns
RXRβ	0.41 ± 0.0	0.88 ± 0.0	<0.01	0.10 ± 0.0	0.11 ± 0.0	ns

p-levels were obtained for the corresponding CO vs. VAD comparisons.

ns: not significant.

Rhythm parameters of circadian retinoic acid receptors protein oscillating levels in the hippocampus of CO and VAD rats.

Table 5

Rhythm Parameters	Mesor (mean ± SE)		Amplitude (mean ± SE)		Acrophase (mean ± SE)	
	CO	VAD	p	CO	VAD	p
RARα	1.15±0.0	1.20±0.0	ns	0.37±0.0	0.26±0.0	ns
RARβ	1.84±0.0	2.00±0.0	<0.01	0.21±0.01	0.07±0.0	<0.01
RXRβ	1.71±0.0	1.32±0.1	ns	0.40±0.2	0.42±0.0	ns

p-levels were obtained for the corresponding CO vs. VAD comparisons.

ns: not significant.

Rhythm parameters of circadian BMAL1 and PER1 mRNA oscillating levels in the hippocampus of CO and VAD rats.

Table 6

Rhythm Parameters	Mesor (mean ± SE)		Amplitude (mean ± SE)		Acrophase (mean ± SE)	
	CO	VAD	p	CO	VAD	p
BMAL1	0.75 ± 0.0	0.60 ± 0.0	ns	0.19 ± 0.0	0.07 ± 0.0	ns
PER1	0.62 ± 0.0	N/A	-	0.20 ± 0.0	N/A	-

N/A: it does not apply, since circadian mRNA levels became arrhythmic.
p-levels were obtained for the corresponding CO vs. VAD comparisons.
ns: not significant.

Table 7

Rhythm parameters of circadian BMAL1 and PER1 protein levels in the hippocampus of CO and VAD rats.

Rhythm Parameters	Mesor (mean ± SE)		Amplitude (mean ± SE)		Acrophase (mean ± SE)	
	CO	VAD	p	CO	VAD	p
BMAL1	1.99 ± 0.0	1.47 ± 0.0	<0.01	0.03 ± 0.0	0.34 ± 0.0	ns
PER1	2.25 ± 0.0	N/A	-	0.48 ± 0.0	N/A	-

N/A: it does not apply, since circadian protein levels became arrhythmic.

p-levels were obtained for the corresponding CO vs. VAD comparisons.

ns: not significant.