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Circadian rhythms of locomotor activity and hippocampal clock genes expression are dampened in vitamin A–deficient rats

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ABSTRACT

The main external time giver is the day-night cycle; however, signals from feeding and the activity/rest cycles can entrain peripheral clocks, such as the hippocampus, in the absence of light. Knowing that vitamin A and its derivatives, the retinoids, may act as regulators of the endogenous clock activity, we hypothesized that the nutritional deficiency of vitamin A may influence the locomotor activity rhythm as well as the endogenous circadian patterns of clock genes in the rat hippocampus. Locomotor activity was recorded during the last week of the treatment period. Circadian rhythms of clock genes expression were analyzed by reverse transcription–polymerase chain reaction in hippocampus samples that were isolated every 4 hours during a 24-hour period. Reduced glutathione (GSH) levels were also determined by a kinetic assay. Regulatory regions of clock PER2, CRY1, and CRY2 genes were scanned for RXRE, RARE, and RORE sites. As expected, the locomotor activity pattern of rats shifted rightward under constant dark conditions. Clock genes expression and GSH levels displayed robust circadian oscillations in the rat hippocampus. We found RXRE and RORE sites on regulatory regions of clock genes. Vitamin A deficiency dampened rhythms of locomotor activity as well as modified endogenous rhythms of clock genes expression and GSH levels. Thus, vitamin A may have a role in endogenous clock functioning and participate in the circadian regulation of the cellular redox state in the hippocampus, a peripheral clock with relevant function in memory and learning.

Abbreviations: ANOVA, analysis of variance; BMAL1, brain and muscle ARNT-Like 1; CAT, catalase; CLOCK, circadian locomotor output cycles kaput; CO, control; CRY, cryptochrome; CT, circadian time; DD, 12-hour dark/12-hour dark; GPx, glutathione peroxidase; GSH, reduced glutathione; LD, 12-hour light/12-hour dark; mRNA, messenger RNA; PER, period; RAR, retinoic acid receptor; RARE, retinoic acid responsive element; RORE, retinoid-related orphan receptor responsive element; RT, reverse transcription; RXR, retinoid X receptor; RXRE, retinoid X responsive element; SCN, suprachiasmatic nucleus; VAD, vitamin A deficiency.

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1. Introduction

Circadian rhythms are biological, physiological, and behavioral oscillations within a 24-hour period and occur in nearly all living organisms, from prokaryotes to humans [1]. In mammals, the endogenous pacemaker that drives circadian rhythms resides in the suprachiasmatic nucleus (SCN) of the hypothalamus. The main exogenous signal that adjusts daily SCN activity is the light-dark cycle. The master clock in the SCN entrains and synchronizes, either by neural and/or humoral signals, the multiple peripheral clocks located in most cells and tissues of the body, including other brain areas [2,3]. Although the most important external time giver (from German: *zeitgeber*) is the day-night cycle, it has been shown that feeding cycles and signals from the activity/rest cycle can also entrain peripheral clocks, independently of light [4,5]. At the molecular level, cellular oscillators consist of a network of interlocking transcriptional-translational feedback loops. The positive limb of the loop is represented by the basic helix-loop-helix PAS transcription factors, Circadian Locomotor Output Cycles Kaput (CLOCK), and Brain and Muscle ARNT-Like 1 (BMAL1) proteins, which heterodimerize and bind to E-box sites in the promoters of the clock negative factors, period (PER1-3) and cryptochrome (CRY1-2) genes [2,6]. A negative feedback loop is achieved when the PERs and CRYs form heterocomplexes that translocate back to the nucleus and inhibit their own and other clock-controlled genes' transcription [7].

It has been reported that vitamin A, through its specific nuclear receptors, may act as a clock activity regulator, one such example by modulating the BMAL1/CLOCK complex binding to the DNA [8]. Interestingly, recent research shows that supplementation of the diet with all-trans-retinoic acid, the vitamin A active metabolite, modifies the phase and amplitude of circadian clock genes expression rhythms in the mouse liver [9]. Inversely, we observed that feeding rats with a vitamin A–free diet altered the daily rhythms of clock BMAL1 and PER1 proteins in the hippocampus as well as in the liver of animals maintained under a 12-hour light/12-hour dark (LD) daily schedule [10,11]. Retinoic acid receptors, RARα, RARβ, RXRβ, and RXRγ have been detected in the hippocampus, and we have also shown that their messenger RNA (mRNA) and protein levels oscillate in a circadian fashion in this brain area [10,12,13]. In addition, we previously showed the rat daily locomotor activity rhythm, a direct outcome of the master clock in the SCN [4], was dampened in the vitamin A deficiency (VAD) [11]. Moreover, some studies have reported a relationship between the circadian clock activity and the cellular redox state [14,15]. Reduced glutathione (GSH), the substrate of the GSH peroxidase system, is a key component in the maintenance of the best redox state for cellular functioning and viability [16]. Previously, we demonstrated that expression and enzymatic activity of antioxidant catalase (CAT) and glutathione peroxidase (GPx) vary on a circadian basis in the hippocampus, being, at least in part, responsible for the circadian oscillation of the cellular redox state in that brain area [10].

In view of the above observations, we hypothesized that the VAD may influence the locomotor activity rhythm as well as the endogenous circadian patterns of clock genes in the rat hippocampus. To test this hypothesis, we aimed to establish nutritional deficiency by feeding the experimental group a diet free of vitamin A during the 3 months of treatment. Our specific goals were (1) to evaluate whether PER2, CRY1, and CRY2 expression and GSH levels displayed an endogenous oscillation in the rat hippocampus; (2) to verify whether locomotor activity exhibited a circadian rhythm; and (3) to assess the effects of the VAD on the circadian locomotor activity rhythms as well as on the endogenous 24-hour patterns of clock genes expression and GSH levels in the hippocampus.

2. Methods and materials

2.1. Animals and diets

Male Holtzman rats, bred in our animal facilities (LABIR, National University of San Luis, Argentina), were weaned at 21 days old and immediately randomly assigned to either the experimental group (standard diet, devoid of vitamin A [VAD group]) or the control (CO) group (standard diet with 4000 IU of vitamin A [8 mg retinol as retinyl palmitate] per kilogram of diet). Diets were prepared according to the AIN-93 for laboratory rodents [17]. The composition (grams per kilogram diet) of experimental and CO diets is shown in Table 1. Animals were maintained under an LD schedule, in a 21°C to 23°C controlled environment, with free access to food and water throughout the 3 months of treatment. To analyze the endogenous circadian rhythmicity, 24 rats from each group were maintained under constant darkness, 12-hour dark/12-hour dark (DD) lighting condition, during the last week of treatment. After the entire treatment period, 4 rats from each group (CO and VAD) were euthanized by CO_2 inhalation; the hippocampus samples were isolated every 4 hours during a 24-hour period and then promptly frozen in liquid nitrogen and stored at –80°C until use. Manipulation of animals in DD was performed under dim red light to avoid acute effects of light. All experiments were repeated a minimum of 2 times. They were conducted in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals [18] and the National University of San Luis Committee's Guidelines for the Care and Use of Experimental Animals.

2.2. Daily locomotor activity analysis

Locomotor activity of individually housed CO and VAD rats was recorded using Achron version 1.3, an acquisition system

Table 1 – Ingredient composition of the diet fed to rats

Ingredients	g/kg diet
Corn starch	397.5
Sucrose	100
Dextrinized corn starch	132
Lactalbumin	200
Soybean oil	70
Cellulose fiber	50
AIN-93 mineral mix	35
10 AIN-93 vitamin mix	10
L-cystine	3
Choline bitartrate	2.5
Tert-butylhydroquinone	0.0014

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for rodent activity, during the last week of treatment under DD conditions. The total daily activity was calculated by averaging the number of infrared beam interruptions/24 hours from each group of animals (n = 4) that were kept in individual cages during the 7 days of activity registration. For each 24-hour cycle, the start and end of the period of activity was determined using the software Simonetta Chronus. The length of the activity period was calculated as the difference between both, and it was expressed in minutes. These determinations were carried out similarly to Allen et al [19]. The corresponding actograms were obtained using the software "The Temps 2.14 version" (www.ub.es/dpfisiv/soft/ElTemps). The periodograms were obtained using the same program, and the endogenous period (T) of each animal was determined.

2.3. *Hippocampus dissection*

Hippocampus samples were isolated every 4 hours, starting at circadian time (CT) 2 (with CT12 defined as the time of the onset of locomotor activity in DD) from CO and VAD groups. Hippocampus isolation was carried out as described in Babu et al [20]. Briefly, following animal decapitation, the head was recovered, and the skull was opened with sterile scissors. The 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.

2.4. *RNA isolation and reverse transcriptase reaction*

Total RNA was extracted from 3 pools of 2 hippocampi each. All RNA isolations were performed using the Trizol reagent (Invitrogen Co), as directed by the manufacturers as previously described [10]. Gel electrophoresis and GelRed staining confirmed the integrity of the samples. Quantification of RNA was based on spectrophotometric analysis at 260 nm. Three micrograms of total RNA was reverse transcribed with 200 units of MMLV Reverse Transcriptase (Promega), using random hexamers in a 25-μL reaction mixture and following the manufacturer's instructions.

2.5. *Polymerase chain reaction amplification*

Transcript levels of CRY1, CRY2, and PER2 were determined by reverse transcription (RT)–polymerase chain reaction and normalized to 28S as endogenous control as described [10]. Fragments coding for those genes were amplified by polymerase chain reaction in 50 μL of reaction solution containing 0.2 mM dNTPs, 1.5 mM $MgCl_2$, 1.25 U of Taq polymerase, 50 pmol of each rat specific oligonucleotide primer, and RT generated complementary DNA (1/10 of RT reaction). The sequences of the specific primers are shown on Table 2. In the case of CRY2 and PER2 genes, samples were heated in a thermal cycler (My Cycler; BioRad) to 94°C for 4 minutes, followed by 35 cycles of (1) denaturation, 94°C for 1 minute; (2) annealing, 56°C during 1 minute; and (3) extension, 72°C for 10 minutes. After 35 reaction cycles, the extension reaction was continued for another 5 minutes. For the CRY1 gene, the thermal cycling conditions were similar but underwent 30 cycles of denaturation-annealing-extension. Polymerase chain reaction products were then electrophoresed on 2% (wt/vol) agarose gel with 0.01% (wt/vol) GelRed. 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 housekeeping 28S gene, which was calculated as the ratio of the mean of gray value of each product to that of 28S.

2.6. *Scanning of clock genes upstream regions for putative RARE, RXRE, and RORE sites*

Putative retinoic acid-responsive (RXRE, AGGTCANAGGTCA), (RARE, AGGTCANNNNNAGGTCA) as well as RORE (A/G)GGTCA preceded by 6 pb A/T) DNA consensus regulatory sites were localized in the regulatory regions of target genes. Thus, up to 2000-bp upstream of the translation start codon of CRY1 (NCBI Gene ID no. 299691), CRY2 (NCBI Gene ID no. 170917), and PER2 (NCBI Gene ID no. 63840) genes were scanned for significant matches using the MatInspector software from Genomatix (http://www.genomatix.de) [21].

2.7. *Reduced glutathione levels*

Reduced glutathione levels were determined in hippocampus samples isolated from CO and VAD rats, following Akerboom and Sies [22]. Briefly, GSH was measured in neutralized acid extracts by a kinetic assay using 5,5′-dithiobisnitrobenzoic acid. Reduced glutathione values were expressed as micromoles per gram of tissue.

2.8. *Statistical analyses*

Time point data were expressed as means ± SEM, and pertinent curves were drawn. Time series were computed first by 1-way analysis of variance (ANOVA) and followed by Tukey post hoc test for specific comparisons; $P < .05$ was considered to be significant. When amplitude or phase was required, a fitting technique was applied. Data were fitted by the following

Table 2 – Primer pairs used for reverse transcription–polymerase chain reaction

Gene name	GenBank accession no.	Forward primer 5′-3′	Reverse primer 5′-3′	Fragment size
CRY1	NC_005106	TCAACAGGTGGCGATTTTTG	TGAAAAGCCTCGGAAACACAT	120 bp
CRY2	NC_005102	CTGGGAGCATCAGCAACACA	GGCAGGCATCTGAGTCACTGT	140 bp
PER2	NC_005108	TGCGGATGTTAGTGGAATCG	GCAGGGAGGTGGACCTTCA	181 bp

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function: c + a cos[2 (t – ø)/24], where c is the mesor, a is the amplitude of the cosine wave, t is time in hours, and ø is the phase in hours from CT0. The fitting was performed using Nonlinear Regression from GraphPad Prism 3.0 software (San Diego, CA). The routine also estimates the SE of the fit parameters. The SE 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 hours of the light regime. The SE of a phase difference was calculated as the square root of the sum of the squared SEs of the differenced phases. In addition 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 hours [23-25]. Briefly, (*a*) in the Cosinor technique, the least squares method served to determine the best fitting cosine function for approximating the data; (*b*) 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 [24]. $P \leq .05$ was taken as indicative of the presence of a rhythm with the 24-hour (anticipated) period. (*c*) Quantitative characterization of rhythms used these 3 main parameters: mesor (24-hour rhythm-adjusted mean: when the interval of time between data sampling is constant, mesor equals the arithmetic mean), acrophase (peak concentration time, is estimated span of time to reach the crest of the detected rhythm for the period [24 hours] under consideration), and amplitude. The Student *t* test was used for comparison of mesor, amplitude, or acrophase between CO vs VAD groups, with $P < .05$ for significant differences.

3. Results

3.1. *Daily locomotor activity*

Given that the locomotor activity rhythm is a direct output of the master clock in the SCN, we started analyzing the activity pattern of rats maintained under constant darkness (DD, free running) conditions and investigated the effects of feeding

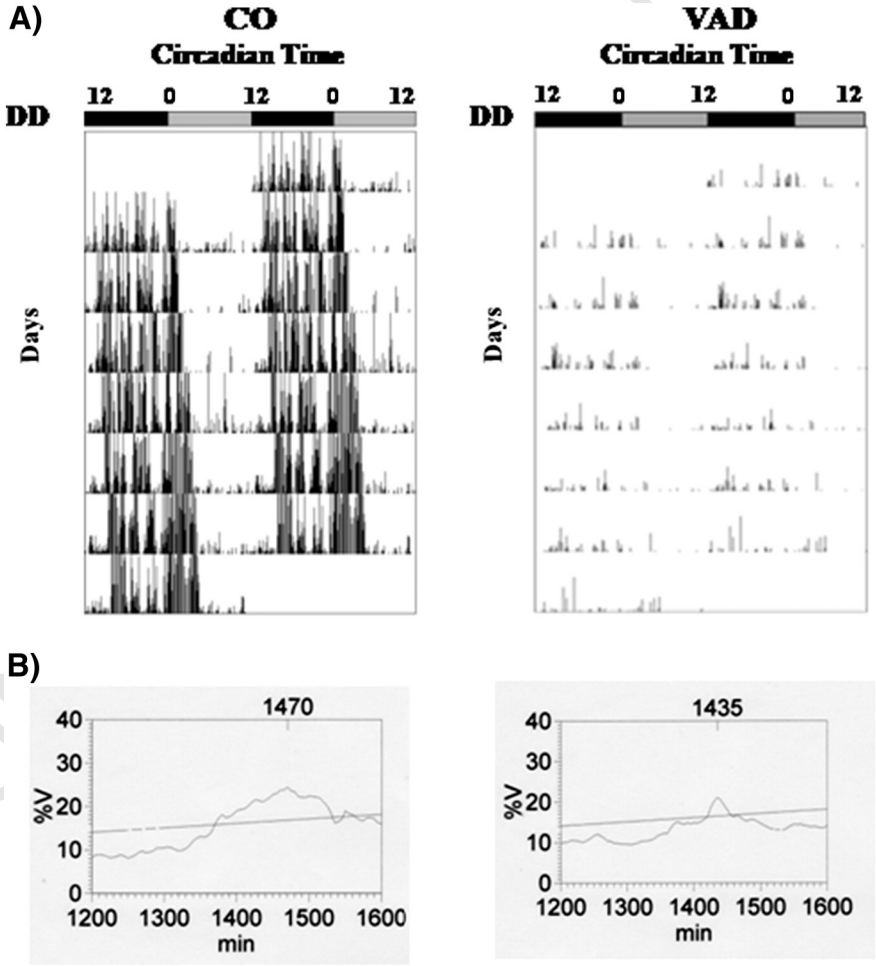

Fig. 1 – Representative actograms of control and VAD rats. A, Double-plot representation of daily locomotor activity of CO and VAD male Holtzman rats, maintained under constant darkness (free running) conditions (top panels, left and right, respectively). In this type of graphic representation, subsequent days are plotted from the top to bottom. Gray and black bars at the top of each actogram represent the distribution of DD phases of a 24-hour photoperiod. B, The lower panels are the corresponding periodograms with circadian period values (ζ), expressed in minutes, above each graph.

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Table 3 – Locomotor activity parameters in DD conditions

Rhythm parameters	CO	VAD
Endogenous period (T)	24:29 ± 00:09 [a]	24:18 ± 00:14 [a]
Duration of the active period (min)	727 ± 12.3 [a]	801 ± 19.3 [a]
Daily total locomotor activity (no. of infrared beam interruptions/day)	2900 ± 395 [a]	890 ± 408 [a]

n = 4 rats per group.
[a] $P < .05$ levels were obtained for the corresponding CO vs VAD comparisons using the Student t test.

them with a vitamin A–free diet. Our analyses revealed that the locomotor activity pattern shifted rightward for the CO rats, indicating an endogenous period (T) greater than 24 hours in these animals (T, 24:29 ± 00:09; Fig. 1A and B and Table 3). Interestingly, a shortened endogenous period (T, 24:18 ± 00:14 vs 24:29 ± 00:09; $P < .05$) was observed in the VAD rats (Fig. 1A and B and Table 3). In addition, the daily total locomotor activity decreased significantly (2900 ± 395 vs 890 ± 408 infrared beam interruptions per day; $P < .05$), whereas the duration of the active period increased (727 ± 12.3 vs 801 ± 19.3 minutes; $P < .02$) in the VAD group (Fig. 2A and B and Table 3).

3.2. *Circadian PER2, CRY1, and CRY2 expression in the rat hippocampus*

We wondered whether PER2, CRY1, and CRY2 genes expression was rhythmic and endogenously driven in the rat hippocampus. To determine this, hippocampus samples were obtained every 4 hours during a 24-hour period from CO rats held in DD. Our results revealed that expression of clock components varies on a circadian basis in the hippocampus. PER2 mRNA levels are maximal at CT 21:42 ± 00:12, whereas CRY1 and CRY2 transcripts peak at CT 09:28 ± 00:18 and CT 05:06 ± 00:55, respectively (Fig. 3A-E and Table 4).

3.3. *Circadian rhythms of PER2, CRY1, and CRY2 expression is modified in the hippocampus of VAD rats*

Having observed PER2, CRY1, and CRY2 expression display circadian rhythms in the hippocampus, we continued to test to what extent VAD could affect the endogenous expression of those key clock factors. Interestingly, feeding rats with a vitamin A–free diet for 3 months phase shifted circadian patterns of PER2, CRY1, and CRY2 mRNA levels (acrophases shifted from CT 21:42 ± 00:12 to CT 16:38 ± 00:34; CT 09:28 ± 00:18 to CT 13:50 ± 00:39, and CT 05:06 ± 00:55 to CT 15:45 ± 01:21; $P < .01$, respectively). In addition, VAD increased the amplitude of PER2 and CRY1 rhythms (0.06 ± 0.01 vs 0.15 ± 0.01, $P < .01$; and 0.05 ± 0.00 vs 0.15 ± 0.02, $P < .01$, respectively) as well as the mesor of PER2 and CRY2 circadian oscillation (0.70 ± 0.01 vs 0.81 ± 0.01, $P < .01$; and 0.48 ± 0.02 vs 0.70 ± 0.01, $P < .01$, respectively; Fig. 3A-E and Table 4).

3.4. *Putative RARE, RXRE, and RORE sites in clock genes regulatory regions*

Once we observed that PER2, CRY1, and CRY2 circadian expression is modified in VAD, we continued to scan regulatory regions of PER2, CRY1, and CRY2 genes to look for retinoid-responsive RARE and RXRE and/or retinoic acid–related RORE sites. The analysis of 2000-bp upstream of translation start codon in the clock genes using the Genomatix database revealed 1 RARE, 1 RXRE, and 2 ROREs in the PER2 regulatory region, whereas 3 RXREs and 1 RORE were found in the CRY1 and 2 RXRE and 1 RORE in the CRY2 genes promoter (Fig. 4).

3.5. *Reduced glutathione expression levels in the hippocampus of VAD rats*

Knowing redox state is essential for cellular clock functioning, we wondered whether GSH levels vary circadianly in the hippocampus. We found GSH levels oscillate on a circadian basis in that brain area, peaking at CT 19:09 ± 00:31, $P < .01$. Vitamin A deficiency reduced the mesor (3.20 ± 0.10 vs 2.34 ± 0.05, $P < .01$), increased the amplitude (0.38 ± 0.05 vs 0.59 ± 0.01, $P < .01$), and phase shifted (CT 19:09 ± 00:31 vs CT 01:50 ± 00:38, $P < .01$) GSH rhythm in the rat hippocampus (Fig. 5 and Table 5).

4. Discussion

Suprachiasmatic nucleus circadian output and the hippocampal endogenous clock are desynchronized in nutritional VAD.

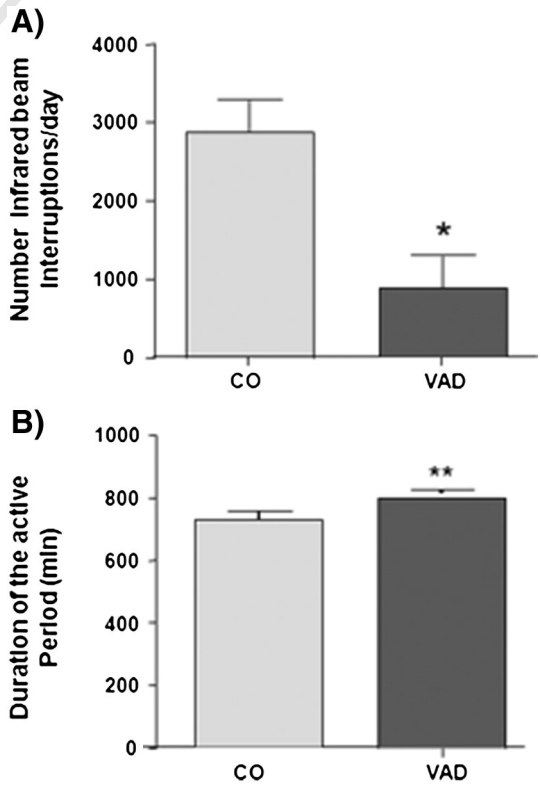

Fig. 2 – Locomotor activity parameters. A, Daily total locomotor activity. B, Duration of the active period of CO and VAD rats. Both groups were maintained under constant darkness (free running) conditions. Statistical analysis was performed by the Student t test, significant differences between groups: $^{*}P < .05$ and $^{**}P < .02$.

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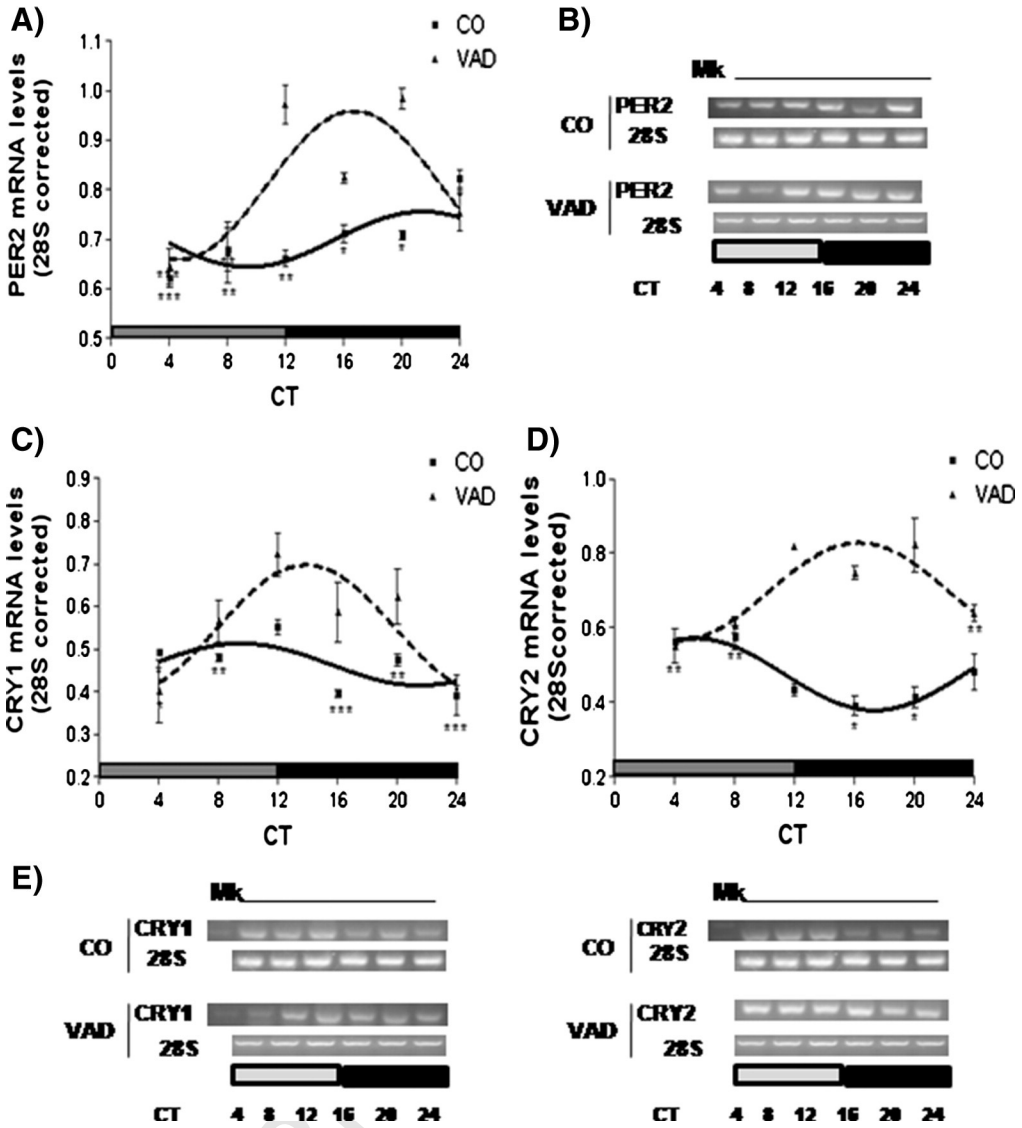

Fig. 3 – Circadian rhythms of PER2, CRY1, and CRY2 mRNA expression in the hippocampus of CO and VAD rats. Cosine fitting curves represent normalized mRNA levels vs CT. Each point represents the means ± SE of 3 pools of 2 hippocampus samples each at a given CT. Horizontal bars represent the distribution of DD phases of a 24-hour photoperiod. Statistical analysis was performed using 1-way ANOVA followed by Tukey test with $^{*}P < .05$, $^{**}P < .01$, and $^{***}P < .001$ when indicated means were compared to the corresponding maximal value in each group. The Cosinor analysis indicated detection of a rhythm with $P \leq .05$ in the mRNA levels. B and E, Representative patterns of polymerase chain reaction products at different CTs in a 24-hour cycle.

It is well known that the activity/rest cycle is under the direct control of the SCN in mammals. Previous studies from our laboratory showed that Holtzman rats display robust daily activity/rest cycles when they are maintained under LD conditions, with the activity period synchronized to the hours of darkness as expected for nocturnal rodents [11].

Table 4 – Rhythms' parameters of circadian PER2, CRY1, and CRY2 mRNA levels in the hippocampus of CO and VAD rats

Genes	Mesor		Amplitude		Acrophase	
	CO	VAD	CO	VAD	CO	VAD
PER2	0.70 ± 0.01 [a]	0.81 ± 0.01 [a]	0.06 ± 0.01 [a]	0.15 ± 0.01 [a]	21:42 ± 00:12 [a]	16:38 ± 00:34 [a]
CRY1	0.46 ± 0.00	0.55 ± 0.04	0.05 ± 0.00 [a]	0.15 ± 0.02 [a]	09:28 ± 00:18 [a]	13:50 ± 00:39 [a]
CRY2	0.48 ± 0.02 [a]	0.70 ± 0.01 [a]	0.10 ± 0.01	0.13 ± 0.02	05:06 ± 00:55 [a]	15:45 ± 01:21 [a]

Values are expressed as means ± SE; n = 4 rats per group.
[a] $P < .01$ levels were obtained for the corresponding CO vs VAD comparisons.

Fig. 4 – Schematic representation of RARE, RXRE, and RORE sites on the 5′ regulatory region of PER2, CRY1, and CRY2 genes. Arrows indicate the first translation codon, gray boxes represent exons, white circles are RARE sites, black circles are RXREs, and gray circles are RORE sites. Negative (–) numbers indicate regulatory sites positions relative to the start of translation (+1).

Other mammals, such as the Northern brown bandicoot [26] and mouse [27] also show locomotor activity rhythms synchronized to LD cycles.

The persistence of rhythms in constant darkness conditions is indicative of endogenous clock control [28]. When we exposed rats to DD for 7 days, their locomotor activity pattern persisted; however, it was shifted rightward in the CO group, thus indicating an endogenous period greater than 24 hours for Holtzman rats. This is consistent with results obtained by Cambras et al [29], Allen et al [19], and Aguzzi et al [30]. The first and the third groups reported endogenous periods greater than 24 hours in Wistar rats, whereas Allen's group did in the Sprague-Dawley rat strain. This would be the first study of locomotor activity rhythms in Holtzman rats; however, taking into account that the Sprague-Dawley is the Holtzman parent strain (Holtzman CO, Madison, WI), it was expected that our results would be similar. Kreze et al [31] show that double knock-out of both murine RXR genes or of either RAR-β/RXR-β or RAR-β/RXR-γ gene combinations exhibits locomotor activity alterations. In addition, Carta et al [32] reported that Sprague-Dawley rats that were maintained on a VAD diet for 6 months exhibited locomotor deficits and impaired motor coordination.

Interestingly, in our experimental model, nutritional VAD dampened rats' daily locomotor activity rhythm, shortened the rats' endogenous period (T), decreased their total locomotor activity throughout the day, and increased the duration of their activity period. Our results are consistent with those observed by Kitaoka et al [33], who reported that nutritional VAD decreased spontaneous activity of mice during a 24-hour period, and also with our previous observations in rats maintained under LD conditions [11]. June et al [34] found that other activity parameters, such as rearing and time of maintenance on the rotarod, also decreased significantly in VAD rats. Since we know that locomotor activity rhythm is a direct output of the master clock in the SCN and the other observations discussed above, it appears that VAD alters the endogenous pacemaker (SCN) activity and might modify the circadian synchronization of peripheral clocks.

Although so far we have not found studies on the consequences of the VAD in the SCN, recently, Meng et al [35] reported the presence of retinoic acid receptors in this brain area, and Guillaumond et al [36] suggested a need for vitamin A in maintaining the signaling downstream of the master clock. Considering that the hippocampus is a peripheral oscillator, which is synchronized by circadian neuroendocrine signals, we continued analyzing the expression of key clock factors in this brain area. In this study, we found that PER2 mRNA levels display an endogenous circadian rhythmicity in the rat hippocampus. This is similar to that observed by Abe et al [37] in the mouse cortex and striatum. In addition,

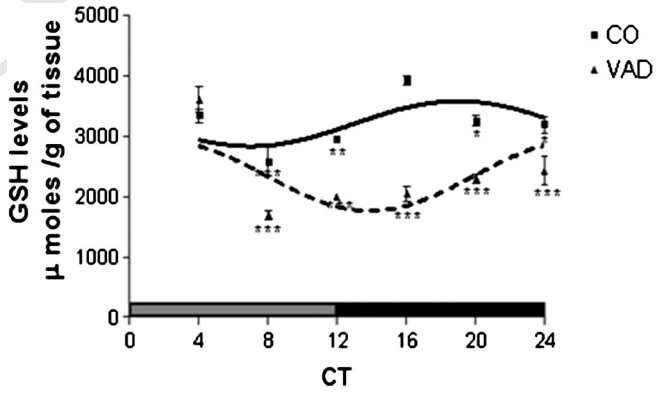

Fig. 5 – Circadian rhythms of GSH levels in the hippocampus of CO and VAD rats. Each point represents the means ± SE of 3 pools of 2 hippocampus samples each at a given CT. Horizontal bars represent the distribution of DD phases of a 24-hour photoperiod. Statistical analysis was performed using 1-way ANOVA followed by Tukey test with $^{*}P < .05$, $^{**}P < .01$, and $^{***}P < .001$ when indicated means were compared to the corresponding maximal value in each group. The Cosinor analysis indicated detection of a rhythm with $P \leq .05$ in the GSH levels.

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Table 5 – Rhythm's parameters of circadian GSH levels in the hippocampus of CO and VAD rats

Rhythm's parameters	CO	VAD
Mesor	3.20 ± 0.10 [a]	2.34 ± 0.05 [a]
Amplitude	0.38 ± 0.05 [a]	0.59 ± 0.01 [a]
Acrophase	19:09 ± 00:31 [a]	01:50 ± 00:38 [a]

Values are expressed as means ± SE; n = 4 rats per group.
[a] $P < .01$ levels were obtained for the corresponding CO vs VAD comparisons.

Mazzoccoli et al [38] also reported endogenous rhythms of PER1-2, CRY1-2 expression in the mouse liver, other peripheral clock. We found that PER2 transcript levels are maximal around the middle of the subjective night in rats. In addition, when we analyzed the expression of CRY1 and CRY2 genes throughout a 24-hour period, we observed that their mRNA levels also oscillate in the hippocampus of rats maintained under free running (DD) conditions. These observations are similar to temporal patterns of clock genes expression observed by Mazzoccoli et al [39] in other peripheral tissues, such as the liver and stomach. Here, we found that CRY1 and CRY2 mRNA levels peak around the middle of the subjective day in the rat hippocampus. Taking into account that feeding cycles, through nutrients, metabolites, and/or metabolism-related hormones, synchronize peripheral clocks in absence of the main *zeitgeber*, the LD cycle [40], a micronutrient, such as the vitamin A, might be essential for the functioning of the endogenous clock. Interestingly, for the first time in our knowledge, we observed that nutritional VAD modifies the endogenous circadian rhythmicity of clock genes expression in the rat hippocampus. Feeding animals with a vitamin A–free diet for 3 months has differential effects on clock genes rhythms' parameters. On one hand, it increased the mesor and the amplitude of PER2 mRNA rhythm but augmented only the amplitude or the mesor of CRY1 and Cry2 transcript oscillation, respectively. On the other hand, VAD delayed PER2 maximal expression while advancing CRY1 and CRY2 mRNA peaks in the rat hippocampus. We and others also reported that VAD modifies the oscillating BMAL1, PER1, and REV-ERBα expression, other key clock factors, in the liver and the hippocampus of rat [10,11,41].

Furthermore, there is evidence that other vitamin deficiencies also result in changes in clock genes expression. For example, vitamin D deficiency, which heterodimerizes with RXR nuclear receptor to regulate the expression of target genes, modifies rhythms of NPAS2 (homolog of CLOCK) and PER2 mRNA levels in rat bone tissue [42]. In addition, it has been reported that folate depletion affects the circadian expression of BMAL1 in mouse liver [43]. Interestingly, our bioinformatic analysis revealed the presence of 1 RARE and 1 RXRE sites within 2000-bp upstream of the translation site in the PER2 gene, whereas 3 and 2 RXRE elements were found in the CRY1 and the CRY2 gene regulatory regions, respectively. In addition, we previously observed that endogenous rhythms of RARα, RARβ, and RXRβ proteins are modified by the VAD in the rat hippocampus [13].

Some studies have reported a close relationship between the cellular clock activity and the local redox state [15,44]. In this study, we determined the levels of a compound of the cellular redox state and a key participant in the cascade of antioxidant defense against oxidative stress, the GSH. It is well known that GSH protects tissues against oxidative damage, either by reducing hydrogen peroxide and organic peroxides in the reaction catalyzed by GPx or by acting as a scavenger of oxygen and nitrogen free radicals. Also noteworthy, daily rhythms of GSH levels have been previously observed in tissues of animals maintained under LD regime [11,45,46], indicating a 24-hour pattern of antioxidant protection. In the present study, we found that GSH levels continue to oscillate in the hippocampus of rats maintained under constant darkness (free running) conditions, thus suggesting that GSH rhythm is circadian and endogenously driven in this memory and learning-related area. In this case, GSH levels are higher around the middle of the subjective night, following previously reported CAT and GPx maximal activity at the beginning of the dark period in the same brain area [10]. Thus, the occurrence of CAT and GPx activity peaks, along with the maximal GSH levels during the night, is important as it would generate a more reduced redox environment and an increased antioxidant protection during the animal activity period. Significantly, this also concurs with the highest level of the brain-derived neurotrophic factor protein and its tyrosine kinase receptor B expression as well as with an increased long-term potentiation in the temporal organization of the rat hippocampus [47-49].

In our model, VAD reduced the mesor, increased the amplitude, and modified the phase of GSH rhythm in the rat hippocampus. Alterations in the circadian rhythmicity of clock factors caused by the VAD might be responsible for changes in antioxidant enzymes circadian expression and activity (previously reported data [50]) and, consequently, in modifying GSH rhythm. Thus, this leads to a poorer antioxidant environment and an increased oxidative stress as well as deleterious effects at the first half of the dark period, which is the time that Winocur and Hasher [51] demonstrated as the most suitable for conducting memory and learning tests in young rats.

This study has 2 notable limitations. First, only male rats were investigated, precluding evaluation of sex differences; however, male rats were selected to obviate potential interactions with ovarian hormones. Second, we investigated 1 strain of rat; the results may not be generalizable to other rat strains.

In conclusion, previous observations and the results of this study support our hypothesis that the nutritional VAD might alter circadian rhythmicity of putative target genes, PER2, CRY1, and CRY2, by modifying the circadian patterns of its retinoic acid receptors expression.

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