Original research paper

Daily rhythms of catalase and glutathione peroxidase expression and activity are endogenously driven in the hippocampus and are modified by a vitamin A-free diet

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Objectives: Alterations in enzymatic antioxidant defense systems lead to a deficit of cognitive functions and altered hippocampal synaptic plasticity. The objectives of this study were to investigate endogenous rhythms of catalase (CAT) and glutathione peroxidase (GPx) expression and activity, as well as *CREB1* mRNA, in the rat hippocampus, and to evaluate to which extent the vitamin A deficiency could affect those temporal patterns.

Methods: Rats from control and vitamin A-deficient (VAD) groups received a diet containing 4000 IU of vitamin A/kg diet, or the same diet devoid of vitamin A, respectively, during 3 months. Rats were maintained under 12-hour-dark conditions, during 10 days before the sacrifice. Circadian rhythms of *CAT*, *GPx*, *RXRγ*, *and CREB1* mRNA levels were determined by reverse transcriptrase polymerase chain reaction in hippocampus samples isolated every 4 hours during a 24-hour period. CAT and GPx enzymatic activities were also determined by kinetic assays. Regulatory regions of clock and antioxidant enzymes genes were scanned for E-box, RXRE, and CRE sites.

Results: E-box, RXRE, and CRE sites were found on regulatory regions of *GPx* and *CAT* genes, which display a circadian expression in the rat hippocampus. VAD phase shifted *CAT*, *GPx*, and *RXRγ* endogenous rhythms without affecting circadian expression of *CREB1*.

Discussion: *CAT* and *GPx* expression and enzymatic activity are circadian in the rat hippocampus. The VAD affected the temporal patterns antioxidant genes expression, probably by altering circadian rhythms of its RXR receptors and clock factors; thus, it would impair the temporal orchestration of hippocampal daily cognitive performance.

Keywords: Antioxidant enzyme, Circadian rhythm, CREB1, Hippocampus, RXR

Introduction

High levels of reactive oxygen species (ROS) in the brain have been associated with impairments in the hippocampal synaptic plasticity.[1,2] It is well known that intense stress response results in higher levels of ROS which, by causing lipid, protein, and nucleic acids peroxidation, can play a role in tissue injury.[3] Particularly, brain tissue is extremely sensitive to oxidative damage because of its large amount of polyunsaturated fatty acids, which are particularly vulnerable to ROS attacks.[4] In order to neutralize ROS, neural tissues bring into play enzymatic, such as glutathione peroxidase (GPx) and catalase (CAT), and non-enzymatic antioxidants, such as vitamin E and glutathione.[5] We, and others, have shown that the nutritional deprivation of vitamin A produces an

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increase in free radicals production and lipid peroxidation and affects the activity of some antioxidant enzymes in several tissues.[6,7]

Numerous studies have shown that alterations in the antioxidant enzymatic defense system produce learning and memory deficits and loss of hippocampal long-term potentiation (LTP).[8,9] Interestingly, both effects have been reversed by the administration of a vitamin A derivative, the retinoic acid (RA).[10]

It is known that vitamin A and its derivatives, retinol and retinal, play a critical role as antioxidants and radical scavengers;[6–11] however, this vitamin has hormonal properties as RA. RA receptors (RARs, α, β, and γ) and retinoid X receptors (RXRs, α, β, and γ) function as ligand-inducible transcription factors.[12] Particularly, RXRγ is highly expressed in a wide range of neuronal tissues, including the hippocampus,[13] and it has been closely related to cognitive deficits in mice.[14,15]

On the other hand, the cAMP response element binding protein (CREB) has been reported to be required for lasting synaptic plasticity associated with long-term memory.[16] CREB is a member of the basic leucine zipper family of transcription factors that binds to the CRE and facilitates the expression of a large and diverse group of genes.[17] It has been demonstrated that CREB1 and RARγ converge in many ways in their signaling. For example, binding of CREB1 to CREs results in the local opening of chromatin and facilitates the access of RARγ to neighboring RAREs and vice versa RARγ seems to help binding of CREB1 to CRE sites.[18] A role for CREB as transcriptional regulator of antioxidant superoxide dismutase gene expression has been described by Bedogni *et al.*[19]

Daily rhythms of CAT, superoxide dismutase, GPx, and glutathione reductase expression and activity have been demonstrated by us, and others, in different brain areas of animals maintained under a conditioned light:dark schedule.[20–22] These variations in the day/night activity of the antioxidant defense system suggest that genes that encode them could be under the endogenous control of the biological clock.

In mammals, the central circadian clock is located in the suprachiasmatic nuclei of the hypothalamus, however, other oscillators have been found in other brain areas and peripheral tissues.[23,24] The molecular clock machinery works through two interacting transcription/translation-based feedback loops, a positive and a negative one. CLOCK (from circadian locomotor output cycles kaput protein) and BMAL1 (from brain and muscle ARNT-like protein 1) constitute the positive elements.[25,26] Upon heterodimerization, BMAL1:CLOCK drives the transcription of the negative components of the clock machinery, three period genes (*PER1*, *PER2*, and *PER3*) and two cryptochrome genes (*Cry1* and *Cry2*) as well as other clock and clock-controlled genes.[27] The PERs and CRYs negatively regulate their own expression, setting up the rhythmic oscillations of gene expression that drive the circadian clock.[28]

Studies in vascular cells suggest that vitamin A and its derivates, the retinoids, may contribute to the regulation of the clock transcription factor activity, through its nuclear receptors RARs and RXRs.[29] On the other hand, Lee *et al.*,[30] have shown that CREB-binding protein also plays a key role in the activation of the BMAL1:CLOCK heterodimer that leads to phase resetting of the circadian clock.

In view of the previous observations, it was of our interest to study the consequences of a vitamin A-depleted diet on the endogenous rhythms of its RXRγ receptor, CREB1, clock factors, BMAL1, and PER1, as well as clock targets, CAT and GPx, in the hippocampus, a peripheral clock with a central role in memory and learning.

Methods

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 assigned randomly to either the experimental diet, devoid of vitamin A (vitamin A-deficient (VAD) group), or the same diet with 4000 IU of vitamin A (8 mg retinol as retinyl palmitate) per kg of diet (control group). Animals were maintained in a 21–23°C controlled environment and 12-hour-light/12-hour-dark schedule, with free access to food and water during the 3 months of treatment. During the last 10 days of the treatment period, 24 rats from each group were maintained under 12 hours dark:12 hours dark (DD) lighting condition. Diets were prepared according to the AIN-93 for laboratory rodents.[31] Both, vitamin A-free 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 (devoid of vitamin A for the vitamin A-free diet), 3 L-cystine, 2.5 choline bitartrate, and 0.014 tert-butylhydroquinone.

After the treatment period, four rats from each group (control and VAD) were sacrificed every 4 hours during a 24-hour period, at the circadian times (CT) CT2, CT6, CT10, CT14, CT18, and CT22 (with CT0 when subjective day starts) for rats in DD condition ($n = 24$). 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 samples were isolated every 4 hours starting at CT2 from control and VAD groups. Hippocampi isolation was carried out as described in Babu *et al.*[32] 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 reaction

Total RNA was extracted from three pools of two hippocampi each. All RNA isolations were performed using the Trizol reagent (Invitrogen, Carlsbad, CA, USA) as directed by the manufacturers. Gel electrophoresis and GelRed™ nucleic acid gel stain (Biotium Inc., San Francisco, CA, USA) 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, Madison, WI, USA) using random hexamers in a 25-µl reaction mixture and following the manufacturer's instructions.

Polymerase chain reaction amplification

Transcript levels of *RXRγ*, *CREB1*, *CAT*, and *GPx* were determined by reverse transcriptase polymerase chain reaction (RT-PCR) and normalized to 28S as endogenous control. Fragments coding for those genes were amplified by PCR in 50 µl of reaction solution containing 0.2 mM dNTPs, 1.5 mM $MgCl_2$, 1.25 U of Taq polymerase, and 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 in Table 1. In the case of antioxidant enzymes expression, samples were heated in a thermalcycler (My Cycler, BioRad, Hercules, CA, USA) to 94°C for 2 minutes, followed by 32 cycles of (1) denaturation, 94°C for 1 minute; (2) annealing, 59°C during 1 minute; and (3) extension, 72°C for 1 minute. After 32 reaction cycles, the extension reaction was continued for another 5 minutes. For *RXRγ* and *CREB1* expression, 35 cycles of amplification were run. PCR products were then electrophoresed on 2% (w/v) agarose gel with 0.01% (w/v) GelRed™ nucleic acid gel stain (Biotium Inc., San Francisco, CA, USA). The amplified fragments were visualized under ultraviolet transillumination and photographed using a Cannon PowerShot A75 3.2 MP 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, calculated as the ratio of the mean of gray value of each product to that of 28S.

Tissue homogenates and enzyme activity assays

Three pools of two hippocampi each, extracted from control and VAD rats at every time point (CT2, CT6, CT10, CT14, CT18, CT22), were homogenized in 1/5 (w/v) dilution in 120 mM KCl and 30 mM phosphate buffer, pH 7.2 at 4°C. Suspensions were centrifuged at $800 \times g$ for 10 minutes at 4°C to remove nuclei and cell debris. The pellets were discarded and supernatants were used to determine antioxidant enzyme activities. The CAT activity was measured by the method of Aebi *et al.*[33] Briefly, 1 ml of supernatant was added to 33 µl of H_2O_2 and the decrease of the absorbance at 220 nm was registered every 5 seconds during a total time of 30 seconds. During this time the decomposition of the H_2O_2 follows a first order reaction kinetic. Determination of GPx activity was based on the spectrophotometric measurement of the rate of NADPH oxidation at 340 nm using tert-butylhydroperoxide as substrate (Flohe and Gunzler's method).[34] The total protein concentration was determined in the enzyme extracts following the Lowry's protocol. All reagents were from Sigma-Aldrich (St Louis, MO, USA).

Scanning of clock-controlled genes upstream regions for putative RXRE, CREB, and E-box sites

Putative RA-responsive (RXRE, AGGTCANAG GTCA) as well as clock-responsive (perfect E-box: CACGTG, or E-box-like: CANNTG) and CREB DNA consensus regulatory sites (CRE: TGACGTAG) were localized in the regulatory regions of target genes. Thus, up to 3000 bp upstream of the translation start codon of *CAT* (Acc no. AH004967) and up to 1500 bp upstream of the same site of *GPx* (Acc no. AB004231), *BMAL1* (Acc no. NC_005100.2), and *PER1* (Acc no. NC_005109.2) genes, were scanned for significant matches using the MatInspector software from Genomatix (Genomatix Software GmbH, Munich, Germany; http://www.genomatix.de).[35]

Statistical analysis

Time point data were expressed as mean ± standard error of the mean (SE) and pertinent curves were drawn. Time series were computed first by one-way analysis of variance followed by Tukey *post hoc* test

Table 1 Primer pairs used for RT-PCR

Gene name	GenBank accession no.	Forward primer 5′–3′	Reverse primer 5′–3′	Fragment size
CAT	NM_012520	CGACCGAGGGATTCCAGATG	ATCCGGGTCTTCCTGTGCAA	174 bp
GPx	NM_030826	CGGTTTCCCGTGCAATCAGTT	ACACCGGGGACCAAATGATG	225 bp
CREB1	NM_031017	TGATGCACCAGGGGTGCCAA	AGTACCCGGCTGAGTGGC	230 bp
RXRγ	NC_005112.2	GCCACCAATAAAGAGAGACT	TCAGCATCTCAAATGGTG	124 bp

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: $c + a\cos[2\,(t–ø)/24]$, 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 (CA, USA). 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 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. Additionally to the significance of conventional statistics, chronobiologic statistics were used for validating temporal changes as rhythms. Thus, each series was analysed by single and population-mean Cosinor method (Halberg Chronobiology Center, MN, USA; http://www.msi.umn.edu/~halberg/) at a trial period of 24 hours.[36–38] 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.[38] A $P = 0.05$ or less was taken as indicative of the presence of a rhythm with the 24-hour (anticipated) period. Student's t-test was used for comparison of mesor, amplitude, or acrophase between control vs. VAD groups, with $P < 0.05$ for significant differences.

Results

Circadian expression and activity of CAT and GPx in the rat hippocampus

Previously, we observed that mRNA levels and activity of *CAT* and *GPx* exhibit daily rhythms in the rat hippocampus, under 12 hours light:12 hours darkness conditions.[22] We wondered whether these expression patterns could be synchronized endogenously. For that, hippocampus samples were obtained every 4 hours during a 24-hour period from control rats maintained under constant darkness (DD) conditions. The results revealed that *CAT* and *GPx* mRNA levels continue oscillating in the absence of light in the rat hippocampus, peaking at the middle of the subjective day (acrophases at CT 06:12 ± 00:13 and 05:45 ± 00:26, respectively; Figs. 1A–D). Consistently, we found that temporal variation of CAT and GPx enzymatic activities follow their oscillating expression and display a circadian rhythm in the rat hippocampus, with their acrophases occurring at CT 13:50 ± 00:29 and CT 11:33 ± 00:52, respectively (Figs. 1E–F and Table 2).

Effect of vitamin A deficiency on circadian rhythms of antioxidant enzymes expression and activity in the rat hippocampus

Circadian oscillating expression of *CAT* and *GPx* was modified by the nutritional vitamin A deficiency in the rat hippocampus. On one hand, the VAD phase-shifted the circadian pattern of *CAT* and *GPx* mRNA (acrophases: CT 06:12 ± 00:13 vs. CT 15:16 ± 00:45, $P < 0.01$; and CT 05:45 ± 00:26 vs. CT 15:37 ± 00:15, $P < 0.01$, respectively; Figs. 1A–D and Table 2). As expected, circadian rhythmicity of CAT and GPx enzyme activity was also phase-shifted in the hippocampus of VAD rats (acrophases: CT 13:50 ± 00:29 vs. CT 04:24 ± 01:12, $P < 0.01$ and CT 11:33 ± 00:52 vs. CT 22:18 ± 02:23, $P < 0.05$, respectively; Figs. 1E–F and Table 2).

Putative RXRE, E-box, and CRE sites on CAT and GPx genes upstream region

Scanning of 3000 bp upstream of the translation start codon in the Genomatix database revealed one RARE, three RXRE, two perfect E-box, three E-box-like, and three CRE sites on the *CAT* gene upstream region, while five RXRE, one perfect E-box, three E-box-like, and three CRE elements were found on the *GPx* gene regulatory region (Fig. 2).

Circadian expression of CREB1 in the hippocampus of vitamin A-deficient rats

We observed that *CREB1* mRNA exhibited circadian expression patterns in the hippocampus of control rats ($P < 0.05$). The highest level of transcript was observed at CT 14:35 ± 00:17 with an amplitude of 0.12 ± 0.00 (Figs. 3A–B, $P < 0.01$ and Table 3). A vitamin A-free diet did not modify the circadian rhythm of *CREB1* expression (Figs. 3A–B and Table 3).

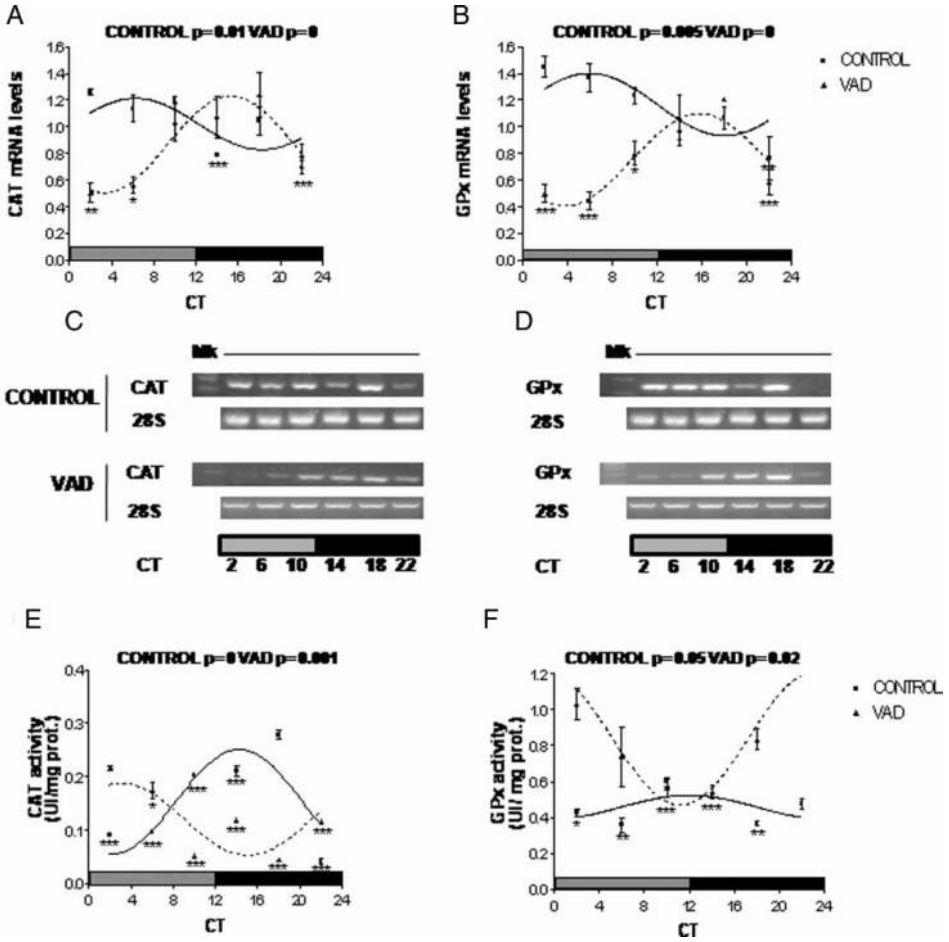

Figure 1 Circadian rhythms of *CAT* and *GPx* mRNA expression and enzymatic activity, in the hippocampus of control and VAD rats. Cosine-fitting curves represent normalized mRNA levels (A–B) and activity (E–F) vs. CT. Each point represents the mean ± SE of three pools of two hippocampus samples each at a given CT. Horizontal bars represent the distribution of dark–dark (DD) phases of a 24-hour period (CT0–CT24). Statistical analysis was performed using one-way analysis of variance 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, $P \leq 0.05$ indicates detection of a rhythm, $P = 0$ indicates $P < 0.0005$ from Cosinor analysis. (C–D) Representative patterns of PCR products at different CTs in a 24-hour cycle. The relative abundance of each band was normalized according to the housekeeping 28S gene.

RXRγ expression levels in the hippocampus of VAD rats

We found *RXRγ* transcript levels show a circadian rhythm in the rat hippocampus ($P < 0.01$) peaking at CT 02:15 ± 00:35 in the control rats. VAD phase-shifted *RXRγ* oscillating expression (acrophase: CT 02:15 ± 00:35 vs. CT 09:09 ± 00:05, $P < 0.01$) without affecting the amplitude and mesor parameters (Figs. 4A–B and Table 3).

Putative RXRE and E-box sites on BMAL1 and PER1 gene upstream region

Looking for putative RXRE and CRE-responsive sites on the clock genes regulatory regions, we scanned 1500 bp upstream of the translation start codon of *BMAL1* and *PER1* genes in the MatInspector database from Genomatix. The search revealed three RXREs and two CRE sites on the *BMAL1* regulatory

Table 2 Rhythms' parameters of circadian *CAT* and *GPx* oscillating expression and activity in the hippocampus of control and VAD rats

	Mesor			Amplitude			Acrophase		
Genes	Control	VAD	*P*	Control	VAD	*P*	Control	VAD	*P*
CAT mRNA	1.02 ± 0.03	0.92 ± 0.12	NS	0.21 ± 0.07	0.41 ± 0.06	NS	06:12 ± 00:13	15:16 ± 00:45	<0.01
CAT activity	0.15 ± 0.00	0.13 ± 0.00	<0.05	0.08 ± 0.02	0.08 ± 0.01	NS	13:50 ± 00:29	04:24 ± 01:12	<0.01
GPx mRNA	0.14± 0.06	0.75 ± 0.05	<0.05	0.27 ± 0.05	0.36 ± 0.03	NS	05:45 ± 00:26	15:37 ± 00:15	<0.01
GPx activity	0.46 ± 0.08	0.73 ± 0.05	<0.01	0.07 ± 0.02	0.31 ± 0.08	<0.05	11:33 ± 00:52	22:18 ± 02:23	<0.05

Note: Data are presented as mean ± SE ($n = 3$ per group). *P*-levels were obtained for the corresponding control vs. VAD comparisons using Student's *t*-test.
NS= not significant.

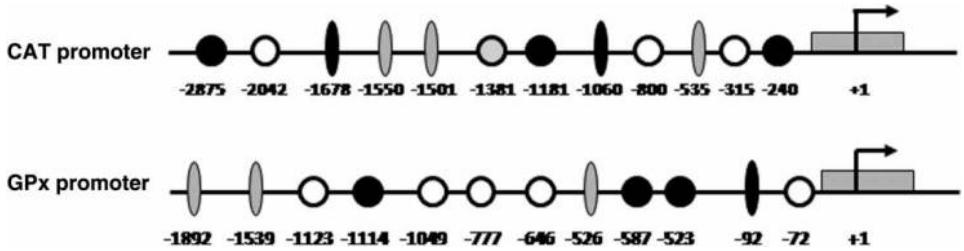

Figure 2 Schematic representation of RARE, RXRE, E-box, and CRE sites on the 5′ regulatory region of *CAT and GPx* genes. Arrows indicate the first translation codon, gray boxes represent exons, white circles are RXRE sites, gray circles are RAREs, black circles are CREs, black ovals represent perfect E-boxes, and gray ovals E-box-like elements. Negative (–) numbers indicate regulatory sites positions relative to the start of translation (+1).

region while two RXREs and three CRE sites were found on the *PER1* promoter (Fig. 5).

Discussion

A variety of clock-driven endogenous rhythms have been reported in the brain. Here, and for the first time in our knowledge, we show that 24-hour rhythms of *CAT* and *GPx* expression are endogenously driven in the rat hippocampus and that their circadian expression patterns are affected in the nutritional vitamin A deficiency.

Even though, previously, some studies show 24 hours variations in the antioxidant enzymes expression and activity in the mammals' brain,[21,22,39] all of them were done in animals maintained under light:dark (LD) conditions; thereafter, none of them was able to report circadian, endogenously driven, rhythms in the hippocampus. Particularly, previous studies from our and Sewerynek's labs demonstrated that, under LD conditions, levels of *CAT* and *GPx* expression and activity vary throughout a 24-hour period in the rat and chicken hippocampus, respectively.[20,22] Such observations led us to question whether those daily oscillations were generated endogenously, in that memory-and-learning-related area. Thus, we continued analysing *CAT* and *GPx* mRNA levels and activity in the hippocampus of animals maintained under constant darkness (DD) conditions. Interestingly, we found that both *CAT* and *GPx* expression and activity display endogenous circadian rhythms in the rat hippocampus. Although we did not find any other study on endogenously generated rhythms of antioxidant enzymes in the mammal hippocampus, circadian patterns of GPx, glutathione reductase, and CAT enzymes activity, were observed by Agapito *et al.*[40] in the cortex of chicks maintained under constant darkness. In this study, *CAT* transcript levels peak around the middle of the subjective day in the rat hippocampus. As we expected, they were followed by the maximal CAT activity, occurring at the beginning of the night (Figs. 1A–F and Table 2). Interestingly, patterns of *GPx* expression and enzymatic activity also continue varying throughout a 24-hour period under DD conditions, in the hippocampus. *GPx* mRNA levels are also maximal around the middle of the subjective day preceding, in 6 hours, the acrophase of its enzymatic activity rhythm (Figs. 1A–F and Table 2). Circadian rhythms of *CAT* and *GPx* expression and activity observed in animals maintained under DD conditions suggest that genes encoding those antioxidant enzymes could

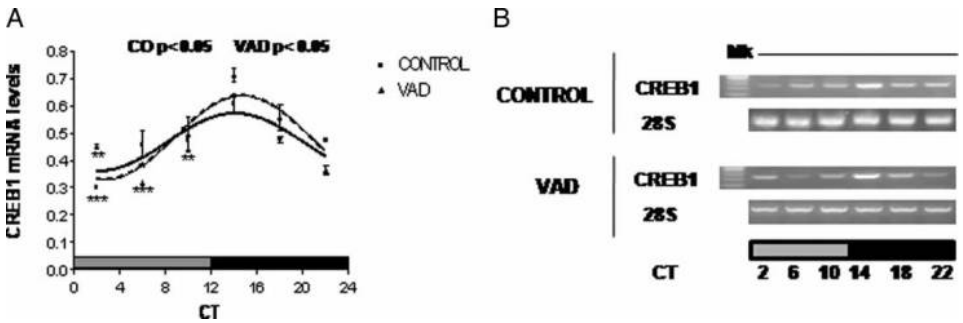

Figure 3 Circadian rhythms of *CREB1* mRNA expression in the hippocampus of control and VAD rats. (A) Cosine-fitting curves represent normalized *CREB1* mRNA levels vs. CT. Each point represents the mean ± SE of three pools of two hippocampus samples each at a given CT. Horizontal bars represent the distribution of dark–dark (DD) phases of a 24-hour photoperiod (CT0–CT24). Statistical analysis was performed using one-way analysis of variance followed by Tukey test with $^{**}P < 0.01$ and $^{***}P < 0.001$ when indicated; means were compared to the corresponding maximal value in each group. Top of the figures, $P \leq 0.05$ indicates detection of a rhythm, $P = 0$ indicates $P < 0.0005$ from the Cosinor analysis. (B) Representative patterns of PCR products at different CTs in a 24-hour cycle. The relative abundance of each band was normalized according to the housekeeping 28S gene.

Table 3 Rhythms' parameters of circadian *RXRγ* and *CREB1* mRNA oscillating levels in the hippocampus of control and VAD rats

	Mesor			Amplitude			Acrophase		
Genes	Control	VAD	*P*	Control	VAD	*P*	Control	VAD	*P*
RXRγ	1.21 ± 0.07	0.99 ± 0.04	NS	0.31 ± 0.03	0.27 ± 0.02	NS	02:15 ± 00:35	09:09 ± 00:05	<0.01
CREB1	0.46 ± 0.00	0.48 ± 0.02	NS	0.12 ± 0.00	0.15 ± 0.02	NS	14:35 ± 00:17	14:46 ± 00:05	NS

Note: Data are presented as the mean ± SE ($n = 3$ per group). *P*-levels were obtained for the corresponding control vs. VAD comparisons using Student's *t*-test.
NS= not significant.

be under the control of the endogenous clock. The circadian expression of clock components, BMAL1 and PER1, as well as RA receptors, RARα, RARβ, and RXRβ, has been assessed in the same brain area, in the DD paradigm.[41]

Interestingly, the bioinformatic analysis revealed us the presence of several clock-responsive, E-box, elements in the regulatory regions of *CAT* and *GPx* genes, suggesting they could be controlled by the endogenous clock. Indeed, and consequently with our previous work,[41] we found maximal *CAT* and *GPx* mRNA expression is in phase with the clock transcriptional activator BMAL1 protein (acrophase at CT 04:31 ± 01:12) and in antiphase with the clock repressor PER1 protein (acrophase at CT: 22:44 ± 00:40). The last finding is consistent with observations made by Hirayama *et al.*,[42] who showed an oscillating pattern of *CAT* expression and activity, in antiphase with the expression of other components of the clock negative circuit, *PER2* and *Cry1*, in Z3 zebrafish embryonic cell line.

Several reports have shown that depletion of vitamin A is associated to alterations in non-enzymatic and enzymatic antioxidant defense systems.[6,22,43] Along with clock-responsive, E-box, elements, our sequence analysis revealed the presence of RXRE sites in the regulatory regions of *CAT* and *GPx* genes (Fig. 2). Transcriptional regulation of *GPx* expression and glutathione homeostasis by the RXRα has been reported by Wu *et al.*[44] On the other hand, it has been demonstrated that RA and its nuclear receptors participate in the modulation of clock activity.[29]

Interestingly, we observed that circadian rhythmicity of antioxidant enzymes is modified in the hippocampus of VAD rats maintained under constant darkness condition. Feeding animals with a vitamin A-free diet, phase-shifted endogenous circadian rhythmicity of *CAT* and *GPx* transcript levels and enzymatic activity. In addition, it modified the mesor of *GPx* circadian expression as well as the mean levels of GPx and CAT activity rhythms, in the rat hippocampus (Figs. 1A–F and Table 2). Even though, to date, we did not find studies on the consequences of VAD on circadian, endogenous, rhythms of antioxidant enzymes, we and others have shown altered patterns of daily expression of those and other enzymes in the VAD.[7,22,45] In a previous study, we observed VAD abolished daily *CAT* mRNA and activity oscillations while phase-shifted *GPx* expression and activity rhythms, in the hippocampus of rats maintained under LD conditions.[22] The discrepancies between circadian (DD) and daily (LD) patterns of *CAT* mRNA levels and enzymatic activity, as well as the different consequences of VAD on them, observed

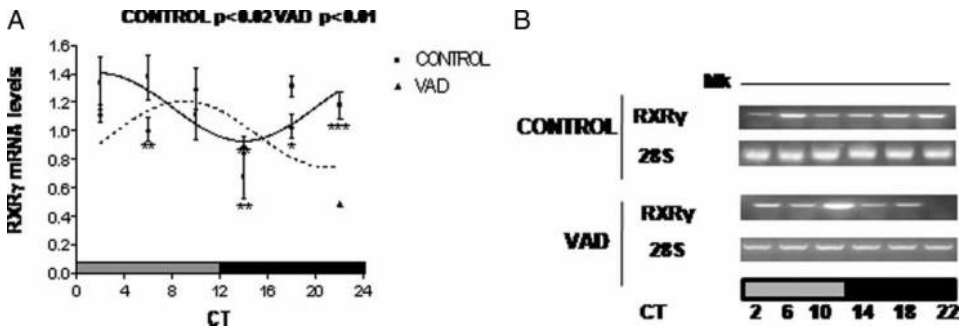

Figure 4 Circadian rhythms of *RXRγ* mRNA expression in the hippocampus of control and VAD rats. (A) Cosine-fitting curves represent normalized *RXRγ* mRNA levels vs. CT. Each point represents the mean ± SE of three pools of two hippocampus samples each at a given CT. Horizontal bars represent the distribution of dark–dark (DD) phases of a 24-hour photoperiod (CT0–CT24). Statistical analysis was performed using one-way analysis of variance followed by the 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, $P \leq 0.05$ indicates detection of a rhythm, $P = 0$ indicates $P < 0.0005$ from Cosinor analysis. (B) Representative patterns of PCR products at different CTs in a 24-hour cycle. The relative abundance of each band was normalized according to the housekeeping 28S gene.

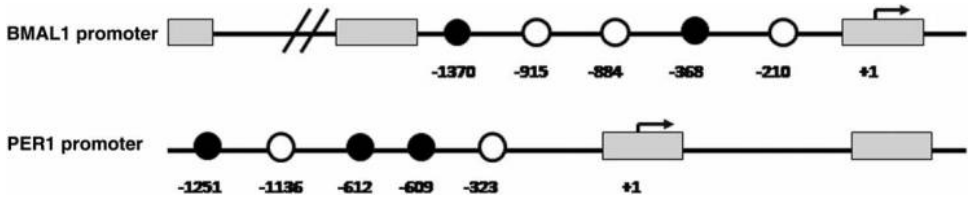

Figure 5 Schematic representation of RXRE and CRE sites on regulatory regions of *BMAL1* and *PER1* genes. Arrows indicate the first translation codon, gray boxes represent exons, gray circles are RXRE sites, and black circles are CREs. Negative (–) numbers indicate regulatory sites positions relative to the start of translation (+1).

here and in our previous work,[22] might be due, at least in part, to differences in their posttranscriptional regulation under different lighting conditions; although masking effects of light on endogenous CAT rhythms might also be occurring in the LD experiment. Evidences of nutritional VAD consequences on 24 hours rhythms have also been reported in other brain areas. For example, when Japanese quails were rendered vitamin A deficient through feeding with a vitamin A-free diet, Fu *et al.*[45] observed that the daily rhythmic expression of the hydroxyindole-*O*-methyltrasferase and arylalkylamine *N*-acetyltransferase (key enzymes in the synthesis of melatonin) almost disappeared in both the pineal gland and retina of the birds. Similarly, Herbert and Reiter[46] observed significant decreases in the amplitude of melatonin and arylalkylamine *N*-acetyltransferase activity rhythms in the pineal of VAD rats.

Knowing the cAMP/MAPK/CREB cascade interacts with the clock to trigger circadian output, that it is involved in memory formation and that CREB1 plays a key role as regulator of antioxidant gene expression,[19,47,48] led us to investigate *CREB1* circadian expression pattern in the rat hippocampus. In fact, our Genomatix analysis revealed the presence of three CRE sites in both *CAT* and *GPx* regulatory regions (Fig. 2). Additionally, when we analyzed the expression of *CREB1* throughout a 24-hour period, we observed mRNA levels oscillate in the hippocampus of animals maintained under free running (DD) conditions (Figs. 3A–B and Table 3). *CREB1* transcript levels are maximal at the beginning of the subjective night period in the control rats, preceding *BMAL1* and *PER1* mRNA peaks (acrophases at CT 15:30 ± 01:15 and CT 17:55 ± 00:21, respectively) observed in our previous work.[41] In this study, we found two CRE sites on the *BMAL1* regulatory intronic region and three CREs on the *PER1* promoter. This would also support a role for CREB1 as transcriptional regulator of the clock as it was demonstrated by Lee.[30] Although, as previously reported, vitamin A deficiency modifies 24-hour rhythms of clock factors in different rat tissues,[7,22,49] it did not modify circadian expression of the clock regulator *CREB1* in this study. The last suggests that transcriptional effects of the nutritional VAD on the clock factors expression, and subsequently, clock-controlled genes expression, would not be mediated by the cAMP signaling pathway. Instead, and consistently with our and others' work, changes in the circadian expression of RARs, observed in the hippocampus of animals fed a vitamin A-free diet, might lead to alterations in the circadian expression of *BMAL1* and *PER1* and, consequently, to changes in their target genes oscillation. Thus, and additionally to circadian rhythms of RARα, RARβ, and RXRβ previously reported[41] here, we also observed *RXRγ* expression displays a circadian profile in the rat hippocampus, peaking at the beginning of the subjective day (Figs. 4A–B and Table 3). Considering it has been demonstrated RXRγ and RXRβ are involved in the LTP, and that this form of synaptic plasticity shows a circadian pattern in different brain areas,[47,50] the rhythmicity of RXRs could constitute, among others, the molecular bases of temporal organization of memory and learning in the hippocampus. Consistently with what we previously observed,[41] the VAD phase-shifted circadian *RXRγ* mRNA rhythm, in this brain area. Thus, we suggest the nutritional VAD might change the circadian rhythms' acrophase of memory-related, RA-target genes, such as the brain-derived neurotrophic factor (BDNF) and neurogranin (*RC3*),[49] by modifying the phase of the circadian patterns of retinoid receptors expression.

In conclusion, here we show that antioxidant enzymes CAT and GPx display endogenous circadian patterns of expression and, consequently, rhythmic enzymatic activity, in the rat hippocampus. Additionally, the expression of *RXRγ* and *CREB1*, is also circadian in the same brain area. As shown in Fig. 6, the temporal occurrence of the endogenous rhythms acrophases follows the order *RXRγ* mRNA, BMAL1 protein, *CAT* and *GPx* mRNA at the beginning of the subjective day followed by CAT and GPx activities, *CREB1* mRNA at the beginning of the night, and PER1 protein at the end of it. The last, as expected, in antiphase with the clock activator BMAL1. The occurrence of maximal CAT and GPx enzymatic activity at the beginning of the subjective night is important since it generates a higher level of

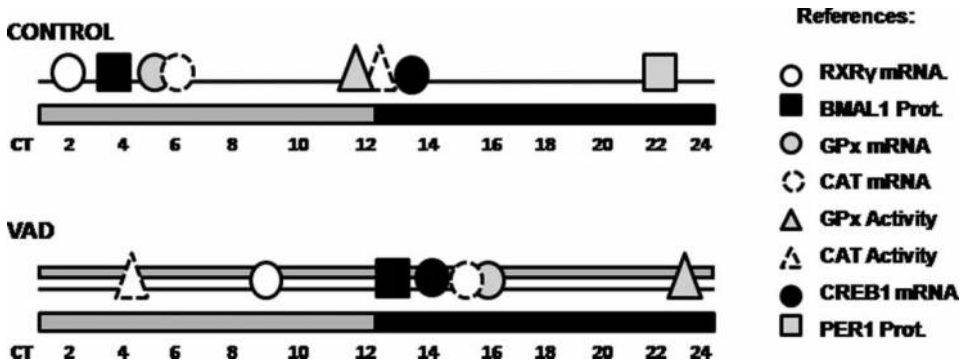

Figure 6 Schematic representation of temporal occurrence of *BMAL1*, *PER1*, *CAT*, *GPx*, *RXRγ*, *and CREB1 rhythms*' acrophases. The circadian distribution of BMAL1 and PER1 proteins, *CAT* and *GPx* mRNA and enzymatic activity, as well as *RXRγ* and *CREB1* transcripts peaks, is represented on a 24-hour temporal line for control and VAD groups. Horizontal bars represent the distribution of dark–dark (DD) phases of a 24-hour period (CT0–CT24). Acrophases symbols' references are indicated on the figure.

reduction and antioxidant protection, preceding peaks of BDNF and its trkB receptor as well as an increased LTP reported previously,[50–52] in the rat hippocampus during the dark phase.

Feeding the animals with a vitamin A-free diet during 3 months modified significantly the phases map. Thus, *RXRγ* maximal expression was delayed 7 hours in comparison to control animals, BMAL1 protein peak was phase-shifted 8 hours and, consequently, *CAT* and *GPx* maximal mRNA levels and activity were also delayed (Fig. 6). These observations suggest that the nutritional VAD would modify the circadian endogenous rhythms of *CAT* and *GPx* at transcriptional level; probably, as a consequence of changes in the temporal patterns of its own receptors and/or the clock factors.

Taking into account that (1) motor as well as cognitive activities occur during the night in rats, (2) the expression of key cognitive factors, such as BDNF, is increased by the nocturnal motor activity and consequently is higher during that period,[51] and (3) some evidence show that synaptic plasticity and cognitive function can be modulated by the cellular redox state and oxidative stress, for example, through the interaction with the BDNF system,[8] the circadian CAT and GPx rhythms, with their higher antioxidant enzymes activity displayed at the beginning of the dark period, might be a mechanism of predictive homeostasis in order to ensure an optimal cellular redox state for the daily cognitive performance in the hippocampus. Interestingly, a nutritional deficiency, such as vitamin A, affected the temporal patterns of clock and antioxidant genes expression, probably by altering circadian rhythms of its RXR receptors, and thus it would impair the temporal orchestration of memory and learning-related tasks in the hippocampus.

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