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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 with 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 *zeitgeber* (from German, *zeit: time and geber:* giver) 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 PER-ARNT-SIM 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, for 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 vitamin A-free diet 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 Archron version 1.3 (Simonetta 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