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Per3 expression in different tissues of *Cebus apella*☆

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ABSTRACT

We present a study of *Per3* expression in six different tissues of the non-human primate *Cebus apella* (capuchin monkey). The aim of this study was to verify whether the expression of the *Per3* gene in different tissues of capuchin monkey occurs in a circadian pattern, its phase and the phase relationships between these different tissues during the 24 h of a day. We observed that gene expression oscillated in all of the tissues studied during this time period, although only the liver and muscle presented a robust circadian pattern. This preliminary study highlights the possibility of using *Cebus apella* as a model to study circadian rhythms at the gene expression level and opens an opportunity for future researches.

1. Introduction

In mammals, the suprachiasmatic nucleus (SCN) of the anterior hypothalamus is the master oscillator that controls circadian output [1,2]. The circadian information from environmental light/dark cycle is received via the retinohypothalamic tract, and thus, the phase of the circadian clock adapts to photoperiods [3]. The SCN coordinates the phasing of myriad circadian oscillators that are present in peripheral tissues [4] to ensure that physiology will be temporally coordinated [5–7].

Among mammals, circadian oscillation is driven by a cell autonomous transcription/translation-based negative feedback loop, wherein the transcription factors CLOCK and BMAL1 form functional dimers and induce the expression of negative regulators (*Per1*, *Per2*, *Per3*, *Cry1* and *Cry2*) that regulate their own expression by inhibiting the CLOCK-BMAL1 complex [8]. Generally, the expression of mammalian clock genes oscillate in a robust circadian manner [1,9,10]. Therefore, the oscillation of the clock genes could be a useful marker for defining the phase [11,12] and angle phase of different peripheral clocks.

The complex of the transcription factors CLOCK-BMAL1 is also responsible for the activation of various clock-controlled genes (CCGs). Thus, the molecular clockwork controls physiological processes through the regulation of CCGs. The clock-controlled genes represent approximately 10% of the expressed genes in a given tissue, and most of these CCGs are tissue-specific because of the different physiological processes carried out in distinct tissues within the appropriate temporal schedule [13,14]. However, the aberrant or desynchronized expression of clock genes within or among individual tissues may have important consequences for the activation of CCGs and, thus, might lead to organ dysfunction [13,15].

Recent studies have suggested that the genetic or functional disruption of certain clock proteins favors the triggering of senescence [16–18] and various physiological disturbances, such as metabolic syndrome [13, 19–21], carcinogenesis [15, 22–24], and cardiovascular diseases [19]. Thus, the desynchronization of the endogenous clock in relation to the environment or between peripheral tissues might affect homeostasis and circadian rhythms regulation [13,15]. These effects can be seen in shift workers and pilots and flight attendants, who are often subjected to transmeridian flights and suffer from higher incidences of cancer [25–29], metabolic pathologies [30–32] and heart diseases [33–37].

The clock gene *Period 3* (*Per3*) has been shown to be associated with Delayed Sleep Phase Syndrome (DSPS) and human chronotypes [38–40]. In humans, this gene presents a tandem 54-nucleotide motif of four or five copies in its coding region. In Japanese and English subjects, DSPS was associated with the allele of this gene with four copies of the motif [38,39], whereas in Brazil, DSPS is associated with the allele with five copies [40]. Recently, it has been shown that this VNTR (Variable Number in Tandem Repeat) polymorphism may profoundly affect sleeping homeostasis and cognitive performance [41–43]. Recently, new roles of *Per3* gene have been proposed in contributing to light input pathways [44] and assisting in timekeeping in the pituitary and lung in mice [45]. Moreover the *PER3* VNTR is a special characteristic of primate molecular clock since this genomic

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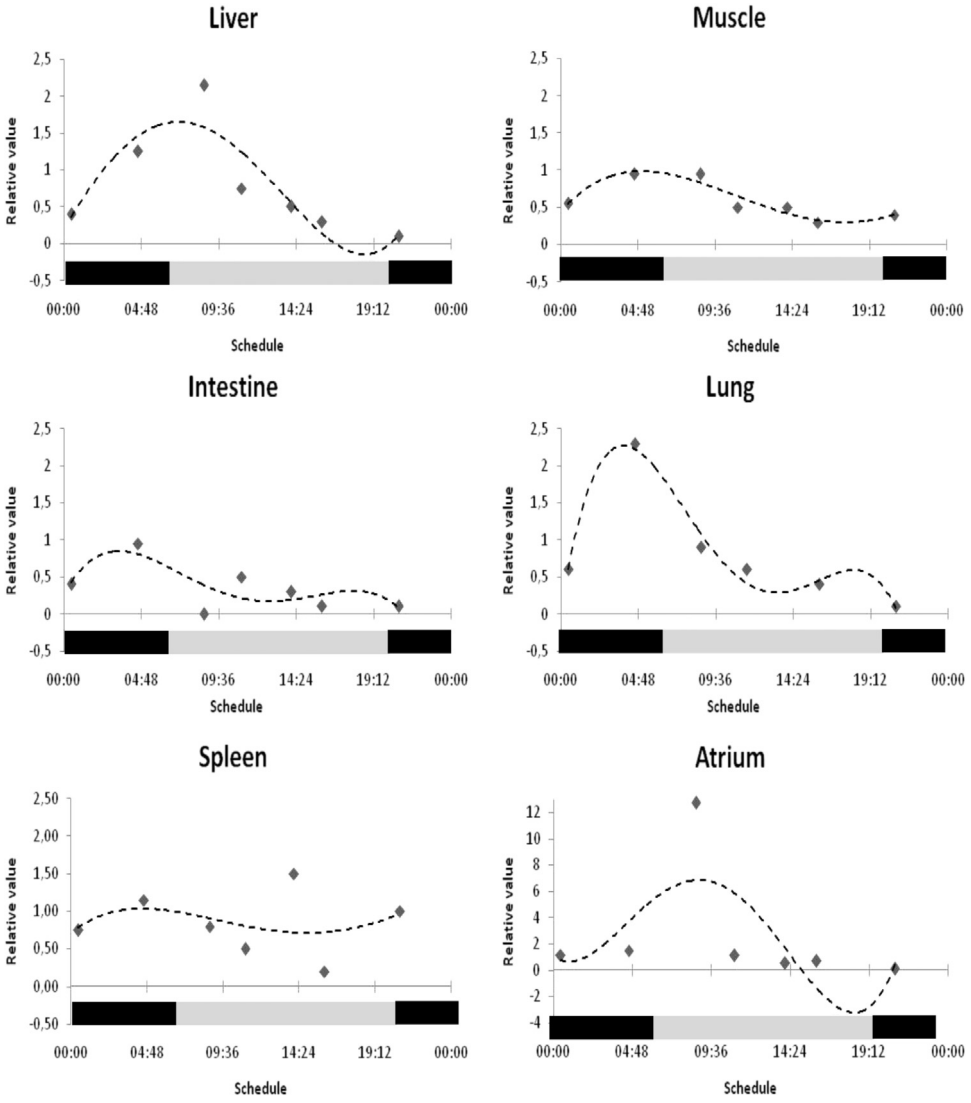

Fig. 1. Daily profile of *Per3* gene expression in the liver, muscle, intestine, lung, spleen and atrium of capuchin monkeys. The animals were sacrificed at different times and on different days, and only one animal was sacrificed for each time point (first day: 8:45, 11:30, 14:05, second day: 8:35, 10:30; third day: 16:00, 20:45, 0:30, 4:40; fourth day: 0:25, 4:30). An average was made of samples collected at successive time points to aid in statistical analysis. The amount of mRNA was assessed by real-time quantification. The time is expressed in hours and the expression data in relative values.

region has not been found in any other mammal species.

Thus, the objective of this study was to verify whether the expression of the *Per3* gene in different tissues occurs in a circadian pattern in capuchin monkeys (*Cebus apella*), and to determine the phases and the phase relationship between these different tissues. Monkeys are adequate models to study human circadian behavior due to the fact that they exhibit diurnal habits [46] and are genetically closer to humans than rodents and fruit flies [47]. Thus, studies using this animal model may lead to new insights into circadian expression pattern of *Per3* in different organs and its relationship with behavior; aiming at the future extrapolation to human health.

2. Materials and methods

Adult *Cebus apella* males from the Tufted Capuchin Procreation Center (UNESP, Campus Araçatuba) were housed in the same conditions, in individual cages and subjected to natural light-dark (LD) cycles. Eleven monkeys were sacrificed at different times (one animal at each time point) on different days (first day: 8:45, 11:30, 14:05; second day: 8:35, 10:30; third day: 16:00, 20:45, 00:30, 4:40; fourth day: 00:25, 4:30). The procedures for animal sedation were performed under red light during the dark phase. Samples of atrium, intestine, liver, lung, muscle and spleen were collected and frozen immediately in dry ice and stored at −80 °C. All experimental protocols of animal used in this study were approved by the Research Committee of the Universidade Federal de São Paulo.

Total RNA was extracted from aforementioned tissues by using a Trizol-based extraction method according to the manufacturer's instructions (Invitrogen, Brazil). Total RNA concentrations were determined by using a spectrophotometer (NanoDrop 8000 Thermo Fisher Scientific), and the quality of RNA samples was assessed by electrophoresis on 1% agarose gels. Total RNA was subsequently treated with DNase I (Invitrogen, Brazil). 1.5 μg of RNA from intestine, liver, lung, muscle and spleen, and 400 ng of RNA from atrium samples were reverse transcribed using SuperScript First-Strand Synthesis for RT-PCR (Invitrogen, Brazil) according to the manufacturer's protocols.

Relative real-time PCR was performed using an ABI PRISM 7500 (Applied Biosystems). The primers were designed with Primer Premier 5 software (PREMIER Biosoft International), and the sequences of the forward and reverse primers were as follows: h*Per3* FW: CAGGCTAACCAGGAATATTACCAGC, h*Per3* RV:

CACAGCCACAGAGAA GGTGTCCTGG; and the *β-actin* primers that were used as the endogenous control were *β-actin* FW: AGGTATCCTGACCCTGAAG, *β-actin* RV: CGTTGAAGGTCTCAAAC ATG. Control PCRs were performed using PCR reaction mixes without cDNA (non-template control) and with a cDNA sample without DNase I treatment.

The Cosinor method was used to verify whether the *Per3* gene expression presented a significant 24-h rhythm in different tissues. The level of significance for detection of the rhythmic cycle was set at $p \leq 0.05$.

3. Results

Per3 expression oscillated over the time period of 24 h in all of the tissues studied. However, significant circadian rhythms were identified only in the liver and muscle according to the Cosinor analysis (with adjusted p-values of 0.046 and 0.028, respectively). The acrophases of *Per3* expression in muscle and liver tissues were 5:58 a.m. and 7:20 a.m., respectively, and the phase angle between these two acrophases is 1 h and 22 min.

The observation of our results reveals that *Per3* gene expression in almost all tissues present the highest level at the end of night and the beginning of morning, although in the spleen, the highest expression level occurs in the middle of night. The lowest level of expression was observed at dusk in the liver and atrium. In the intestine, lung, spleen and muscle, the minimum level of expression occurred in the middle of the afternoon (Fig. 1).

4. Discussion

It has been reported that the expression of several clock genes of different tissues of rodents, such as heart, lung, liver, stomach, spleen, kidney, pancreas, retina, bone marrow, submandibular gland and skeletal muscle exhibits a circadian pattern [48–57]. On the other hand, testis and thymus appear to have no oscillating pattern of the expression of clock genes [58,59]. In humans, most studies have shown robust circadian expression of clock genes in peripheral blood and tumor cells [23,24,60,61]. However, to date, there are few studies on the expression of clock genes in peripheral tissues of primates [62].

Although we were able to show that *Per3* is expressed in six different tissues of a non-human primate (capuchin monkey), our study has some limitations: the data were collected from only one animal at a time, thus increasing the effects of individual variability, and we collected the samples for only one period of 24 h. In spite of these limitations, it was possible to observe circadian expression of the *Per3* gene in the liver and muscle, which indicates that the 24-h oscillation is clearly detectable.

Another study showed that *Per3* expression in skeletal muscles of mice presented a peak of expression between the end of a subjective day and the beginning of a subjective night [51]. The *Per3* expression pattern that we observed in *Cebus* monkeys is in opposite phase compared to these previous results. This result is expected because of the antiphase expression of circadian rhythms behaviors in monkeys and mice, which exhibit diurnal and nocturnal activity preferences, respectively.

A recent study evaluated the profiles of periodic gene expression in peripheral blood mononuclear cells of young and old people [61]. The *Per3* expression peak of the individual daily profiles occurred at the beginning of the morning or the end of night, as we also observed in the liver and muscle in capuchin monkeys. They showed that the acrophase for *PER3* was at 08:28 ± 00:37 for older subjects and 05:57 ± 00:26 for young controls. Although the tissue was not the same we analyzed, the data are very similar to those obtained in this study in monkeys in terms of phase, as we found that the acrophase was at 5:58 a.m. in muscle and at 7:20 a.m. in liver. In that way the *PER3* phase of expression or phase relationships between tissues could be used as an index of circadian entrainment.

To the best of our knowledge, this study is the first to show the expression of the *Per3* gene in six different tissues of a non-human primate. We found a robust circadian rhythm in the liver and muscle. However, it is not possible to assume whether the absence of 24 h rhythms observed in the other tissues was attributable to the experimental design, or if there are actually no robust circadian rhythms in these tissues. The present results suggest that *Cebus apella* represents a suitable model for studies of circadian oscillations, but more elaborate studies are needed to understand the relationship between the *Per3* circadian phase, the phase angle in different tissues and circadian behavior. It is necessary to understand these circadian patterns of expression in each tissue to better understand disorders that are caused by the disruption of circadian rhythms.

The pattern of expression of clock genes allows for each organ to be able to respond to various physiological stimuli that change throughout the day. Therefore, the alteration of this temporal order will have physiological consequences, making it critically important to understand the role of *Per3* and other circadian clock genes in the physiology of each organ.

The human *PER3* gene is associated with myriad of sleep and circadian phenotypes and also with disease states [40,63,64], mainly a functional genomic structure, a VNTR in exon 18th, that has been demonstrated to be exclusively a primate region [65] which could be associated with particular aspects of sleep and circadian rhythms evolution in this Order of animals. The *Cebus appela* particulary has the same structure, the VNTR, in its *Per3* structure but it is a little beat different of humans because it has only two repeats and it is not polymorphic. It remains to be clarified if the number of repeats in the *Per3* VNTR in different primate species has some particular role on circadian processing that is specie-specific.

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