The pineal gland is not essential for circadian expression of rat period homologue (rPer2) mRNA in the suprachiasmatic nucleus and peripheral tissues

Katsutaka Oishi\textsuperscript{a}, Ichiro Murai\textsuperscript{b}, Katsuhiro Sakamoto\textsuperscript{a,c}, Hitoki Otsuka\textsuperscript{d}, Yoshiaki Miyake\textsuperscript{d}, Takahiro Nagase\textsuperscript{e}, Norio Ishida\textsuperscript{a,f,*}

\textsuperscript{a}Ishida Group of Clock Gene, National Institute of Bioscience and Human Technology, Agency of Industrial Science and Technology, MITI, Tsukuba, Ibaraki 305-8566, Japan
\textsuperscript{b}Department of Biochemistry, Nihon University School of Medicine, Itabashi, Tokyo 173-0032, Japan
\textsuperscript{c}Japan Science and Technology Corporation, Japan
\textsuperscript{d}Department of Obstetrics and Gynecology, Nihon University School of Medicine, Nerima, Tokyo 179-0072, Japan
\textsuperscript{e}Kazusa DNA Research Institute, Kisarazu, Chiba 292-0812, Japan
\textsuperscript{f}Department of Biomolecular Engineering, Tokyo Institute of Technology, Yokohama, Kanagawa 226-8501, Japan

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Abstract

To investigate the functional involvement of the pineal gland in circadian expression of the rat period homolog gene (rPer2) in the suprachiasmatic nucleus (SCN) and peripheral tissues, we performed Northern blot analysis in tissues from pinealectomized rats. The ectomy did not have any significant effects on rPer2 mRNA expression patterns both in a daily light-dark condition and in a constant darkness. These results suggest that the rhythmic secretion of pineal melatonin is not essential for the circadian expression of clock genes in the SCN and other peripheral tissues of rats. © 2000 Elsevier Science B.V. All rights reserved.

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Mammalian homologs of Drosophila clock gene period (per) are thought to be the essential elements that regulate circadian rhythms in organisms [7]. Recently, Zheng et al. [27] showed that a deletion mutation in the PAS domain of mPer2 gene abolished a circadian rhythmicity of locomotor activity in mice. This data define mPer2 as a component of the mammalian circadian oscillators. Mammalian per homologs (per1, per2 and per3) and BMAL1 (a putative clock gene which encodes a basic helix-loop-helix PAS transcription factor) mRNAs exhibit a robust circadian expressions in the suprachiasmatic nucleus (SCN): the principal pacemaker that regulates various physiological and behavioral circadian rhythms in mammals [7]. These mRNAs are rhythmically expressed even in peripheral tissues such as eye, heart, lung, spleen, kidney and liver [17–19,23,28]. Previously, we showed that the circadian expression of rPer2 mRNA in peripheral tissues is governed by the SCN, because the rhythmic expressions are abolished in SCN-lesioned rats [23]. We also suggested that the humoral factors, driven by the SCN, mediate the circadian expression of per genes in peripheral tissues, because the rhythmic expression of per genes are observed in circulating mononuclear leukocytes that have no neuronal connections [18]. In vitro experiments using rat-1 fibroblast culture systems have also implied the existence of humoral factors that regulate the circadian expression of clock genes in peripheral tissues [2,25]. However, little is known about the factors mediating the rhythmic expression of mammalian clock genes.

In mammals, the pineal hormone melatonin (N-acetyl-5-
methoxytryptamine) is involved in the regulation of numerous physiological and behavioral processes cued by the environmental light–dark cycle [20]. Although pinealectomy has no significant effect on free-running locomotor rhythms, exogenous melatonin modulates the function of the circadian clock in the SCN, and entrains the activity and sleep in mammals [1,4]. Circadian synthesis of pineal melatonin is endogenously controlled by the SCN and is synchronized by environmental light [20]. While the SCN is a major site of melatonin binding in the mammalian brain, melatonin binding sites at the periphery have also been detected [3,5,16]. Thus, it is reasonable to speculate that circulating melatonin from the pineal gland mediates the circadian expression of mammalian clock genes in peripheral tissues.

In this study, to investigate the functional involvement of rhythmically secreted pineal melatonin in the expressions of mammalian clock genes in peripheral tissues, we examined the temporal expression patterns of rPer2 mRNA in tissues from pinealectomized rats under a daily light–dark cycle and under constant darkness by Northern blot analysis.

Male Wistar rats (8-weeks-old) obtained from Clea Japan, Inc. (Tokyo) were pinealectomized as described [14]. The rats were housed under a 12 h light–12 h dark cycle [LD 12:12; lights on at Zeitgeber time (ZT) 0]. A white fluorescent lamp was used as a source of light during the day (150–200 lux at the level of the cages).

We measured blood melatonin concentrations of rats under LD 12:12, to confirm that the operation for pinealectomy had been completely successful. Two weeks after the operation, we collected blood samples using a heparinized microcapillary from the tail vein. The plasma fraction was gained by centrifugation, then the circulating melatonin levels were measured with commercially available ELISA kit (Bühlmann Laboratories Ag, Allschwil, Switzerland) that has a sensitivity of 3.6 pg/ml. When we drew the blood at night, a dim red light was used in order to avoid a possible light influence on the melatonin profile.

Four weeks after the operation, rats were decapitated, and tissues were dissected, quickly frozen, and kept in liquid nitrogen until used. In darkness, dissections were carried out under a dim red light. To investigate whether the expression of rPer2 mRNA was influenced by the masking effect of light, we examined the effect of pinealectomy under a constant darkness (DD) as well as in an LD condition. The experiment in DD was performed after free-running for 2 days in DD. Total RNA was isolated from tissues and separated on a 1% agarose/0.7 M formaldehyde gel as described [22]. RNA was transferred to a nylon membrane (GeneScreen Plus; DuPont, USA) by passive capillary transfer. Each lane contained 20 μg of total RNA from each tissue or 5 μg of total SCN RNA. A 32P-labeled random primed probe was generated from a rPer2 cDNA fragment (bases: 1144–1797; GenBank accession number AB016532), then hybridization and detection were performed as described [22]. Relative mRNA levels were normalized by determination of the amount of β-actin mRNA (data not shown).

At first, we measured blood melatonin concentrations of rats under LD 12:12, to confirm that the operation for pinealectomy had been achieved completely. The robust daily secretion of plasma melatonin was abolished by theectomy (Fig. 1). The small amount of circulating melatonin in the pinealectomized rats might have been secreted by retina and other peripheral organs, since there is accumulating evidence showing that the mammalian retina [9,21,22,26] and peripheral blood mononuclear leukocytes [10] can synthesize and even secrete melatonin.

We examined the rPer2 mRNA expression in whole brain, eye, and heart both in LD 12:12 (Fig. 2). In pinealectomized rats as well as in sham control rats, the robust circadian expression of rPer2 mRNA with a peak between ZT 14 and 17, was observed in each tissue. We also examined the mRNA expression patterns of rPer1 and BMAL1 genes, and observed that the pinealectomy had no significant effect on the mRNA expression patterns in each tissue (data not shown).

To evaluate whether the diurnal fluctuation of rPer2 mRNA is a response to a light–dark periodicity or reflects a circadian clock, we examined the mRNA levels under DD (Fig. 3). However, the rhythmic expression of rPer2
Fig. 2. Daily expression of rPer2 mRNA in the SCN (n=3), whole brain (n=1), and peripheral tissues (eye: n=3, heart: n=1) from pinealectomized or sham rats. (a) Representative Northern blot analyses of rPer2 mRNA. Rats were housed in LD 12:12 (lights on at ZT 0) for 4 weeks after the ectomy. The animals were sacrificed at each time-point indicated above the horizontal bar. The open bar indicates lights-on, and the dark bar indicates lights-off. (b) Quantification of rPer2 mRNA expression from the Northern blots. Filled circles indicate values from the pinealectomized group and open circles from the sham group. The maximum value of the sham group was expressed as 100% in each tissue.

mRNA was observed in pinealectomized rats as well as in sham control rats. The ectomy did not cause any significant differences between the groups.

In this study, we showed that the pineal gland is not indispensable for the rhythmic expression of mammalian clock genes in rat tissues. This is the first report that described the relationship between the pineal gland and the circadian expression of clock genes in rats.

A number of studies have indicated that the administration of exogenous melatonin can entrain the circadian locomotor patterns of mammals [1,4]. Since cyclic AMP (cAMP)-mediated mechanisms are important for the phase-resetting of circadian systems [12], the entraining effects of exogenous melatonin seem to involve the regulation of cAMP accumulation via the G protein-coupled receptors [11,13,15]. Recently, in vitro experiments using rat-1 fibroblasts have implied the involvement of cAMP/protein kinase A signaling pathway in the regulation of rPer1 expression [25]. Although the entraining mechanism of exogenous melatonin is still unknown, the exogenous melatonin might change the expression levels of clock genes, and consequently entrain the circadian rhythms of mammals. However, our results suggest that the physiological pineal melatonin is not essential for the circadian expression of clock genes either in the SCN or in peripheral tissues.

As described above, we previously suggested that in mammals the SCN governs the circadian expression of per
Fig. 3. Circadian expression of rPer2 mRNA in the SCN (n=2), whole brain (n=1), and peripheral tissues (eye: n=2, heart: n=1) from pinealectomized or sham rats in constant darkness. (a) Representative Northern blot analyses of rPer2 mRNA. Rats were housed in LD 12:12 (lights on at ZT 0) for 4 weeks after the ectomy, then transferred into constant darkness (DD). The animals were sacrificed at each time-point indicated above the horizontal bar. The shaded bar indicates subjective day, and the dark bar indicates subjective night. (b) Quantification of rPer2 mRNA expression from the Northern blots. Filled circles indicate values from the pinealectomized group and open circles from the sham group. The maximum value of the sham group was expressed as 100% in each tissue.

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References


