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Circadian rhythms in rat pineal melatonin synthesis under constant darkness conditions

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Abstract: To investigate whether rat pineal cell is capable of generating a circadian oscillation in melatonin release, the cultured pineal cell in culture medium was collected at 6∶00, 8∶00, 10∶00, 12∶00 and 15∶00 during the day, and at 18∶00, 20∶00, 22∶00, 1∶00 and 4∶00 at night respectively. The melatonin level in culture medium was measured by a direct radioimmunoassay. The complex cosine functions (CCF) statistical analysis indicates that the rat pineal cell melatonin synthesis shows an intrinsic rhythm at 3, 6, 9 day culture under constant dark conditions. However, the intrinsic rhythm disappears at 12 day culture. This result suggests that the rat individual pineal cells can function as a circadian oscillator in vitro.

Key words: rat; pineal; cell culture; melatonin; constant darkness; circadian
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持续黑暗状态下对大鼠松果体细胞分泌松果体素的昼夜节律研究

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摘要:为研究体外培养的大鼠松果体细胞分泌的松果体素是否具有内在节律,收集体外培养的大鼠松果体细胞在白天6∶00,8∶00,10∶00,12∶00,15∶00,和夜晚18∶00,20∶00,22∶00,1∶00,4∶00各个时间点的培养上清,并用放射免疫方法(RIA)检测各个时间点松果体细胞培养上清中的松果体素水平. CCF (complex cosine function)统计分析结果表明,在持续黑暗的环境下,体外培养的大鼠松果体细胞分泌的松果体素在培养3,6,9天后仍然表现出内在节律. 然而,这种内在节律在培养12天后消失. 上述结果提示体外培养的大鼠松果体细胞可以作为一个有用的体外模型来分析节律系统的生理基础.

关键词:大鼠;松果体;细胞培养;松果体素;持续黑暗的环境;节律

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0 Introduction

Melatonin is mainly synthesized in the pineal gland and it plays an important role in the regulation of circadian rhythms under light/dark (LD) cycles[1-2] and other physiological and pharmacological functions[3-5]. Melatonin is synthesized from tryptophan (Trp) by a series of catalyzing enzymes. First, tryptophan (Trp) is taken up from the circulation and is both hydroxylated and decardoxylated to produce serotonin catalyzed by both tryptophan hydroxylase (TPH) and aromatic L-amino acid decarboxylase (AADC). Then, serotonin is catalyzed by the N-acetyltransferase (NAT) to N-acetyl-5-hydroxytryptamine, which is in turn catalytically converted by hydroxyndole-O-methyltransferase (HIOMT) to melatonin. Following its synthesis, pineal melatonin is passively secreted into the circulation[6]. Melatonin production increases in the dark and is inhibited by light. Light plays a role in the control of the mammalian pineal melatonin rhythm. In addition, the SCN (suprachiasmatic nucleus) is the major circadian pacemakers in mammals[7]. The sequence of events leading to generation of the pineal melatonin rhythm includes the perception of the LD cycle by the eyes, entrainment of the clock in the SCN, and the generation of a circadian pattern of neural activity in the sympathetic input to the pineal, leading to increased norepinephrine (NE) release at night. NE binding to α- and β-adrenoceptors initiates a chain of events beginning with enhanced cyclic adenosine monophosphate (cAMP) synthesis[8-9]. As a second messenger, cAMP regulates pineal NAT and ultimately melatonin biosynthesis in two ways. First, NAT activity is stimulated by a cAMP-dependent mechanism involving both the transcription and translation. Second, cAMP acts in some way to prevent the inactivation of NAT[10]. The rhythm of NAT activity is believed to be responsible for the rhythm in melatonin synthesis.

Several experiments have demonstrated that the rhythm of the chick pineal cells persisted under constant darkness and maintained for several days when the gland is under the culture condition in vitro[11-12]. The melatonin rhythm generating system in the chick is distinctly different from the rat. The chick pineal rhythm in melatonin synthesis is regulated by two oscillators, one in the pineal gland itself[13-14] and the other in the SCN[15]. Although two clocks are involved in vivo, the clock in the pineal cell is sufficient to generate a rhythm in NAT activity in vitro.

Studies have demonstrated that the individual lizard pineal cells rhythmically produced melatonin in either LD cycle conditions or in constant darkness. In addition, it has been reported that melatonin production from individual lizard pineal cells showed daily fluctuations, which indicates that the individual lizard pineal cells can function as circadian oscillators[16-17].

Recently, it was reported that turkey pineal gland and retina showed diurnal and circadian rhythms in melatonin synthesis[18-19]. They reported that the circadian rhythms in NAT activity and melatonin concentrations were found both in the turkey pineal gland and retina under constant darkness (DD) conditions. However, the enzyme activity of the hydroxyindole-O-methyltransferase (HIOMT) in both the pineal gland and retina did not show significant changes throughout the 24 h period.

The purpose of this study is to test whether the rat individual pineal cell shows circadian rhythm of melatonin production in vitro. The complex cosine function (CCF) statistical analysis indicates that the intrinsic rhythm of the neonatal rat pineal cell melatonin synthesis is maintained for several days under culture condition in constant darkness. The data should provide a useful model in vitro to analyze the physiological basis of circadian system.

1 Materials and methods

1.1 Animals and reagents

Neonatal (1-day-old Sprague-Dawley, SD) rats from the Experimental Animal Center of Anhui Province were used in this study. The parent rats were housed at 20 ~ 23 ℃ under a 12 h∶12 h light/dark cycle (light on at 07∶00; light off at 19∶00). Animal housing, care and application of experimental procedures were in accordance with all relevant local guidelines and legislation to minimize pain and suffering of the animals.

Dubucco's modified Eagle's medium (DMEM) and fetal bovine serum (FBS) were purchased from Gibco. 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), L-glutamine, penicillin, streptomycin, rabbit anti-serotonin, collagenase, and Ascorbic acid were obtained from Sigma-Aldrich. Melatonin Research Radioimmunoassay kit was from Labor Diagnostika Nord (LDN, Germany).

1.2 Pineal cell culture

Primary rat pineal cell culture was performed as previously described[20]. Pineal glands were isolated from SD rats under sterile condition on day 1. After incubation with 0.25% trypsin solution at 37 ℃ for 20 min, the dispersed pineal cells were maintained in DMEM supplemented with 10% fetal calf serum. 55 mg/L sodium pyruvate, 0.1 g/L ascorbic acid, 4 g/L glucose, 0.1 mg/L streptomycin, and 100 units/mL penicillin *G* were added to a 6 well cell culture cluster containing 3.0×10^5 cells/mL. Cells were cultured in a humidified 5% CO_2 incubator at 37 ℃ under constant darkness conditions.

1.3 Culture medium collection

The cell culture medium for measuring melatonin dual rhythm circadian after 3, 6, 9, 13 day culture was collected at 6∶00, 8∶00, 10∶00, 12∶00, 15∶00 during the day, and at 18∶00, 20∶00, 22∶00, 1∶00, 4∶00 at night respectively. A total of 11 time points was taken over a 24 h period. Light was avoided when the cell culture medium was collected. The supernatants were centrifuged for 10 min at 12 000 r/min, and the samples were kept at −80 ℃ until assayed.

1.4 Melatonin determination

Melatonin in culture medium was measured by a direct radioimmunoassay (RIA). The assay was conducted according to the procedure of the commercial kit. The standards of melatonin levels measured with the melatonin research RIA kit ranged from 0 ng/L to 1 000 ng/L. The standard curve of melatonin assay was highly reproducible with an average correlation coefficient of 0.999. Each sample was measured in duplicate. The cross reactivity between melatonin and N-acetylserotonin, serotonin was 0.8% and less than 0.01%, respectively. The average intra- and inter-assay coefficient of variation (CV) was 7.5% and 15.1%, respectively. The sensitivity of the assay is 0.8 ng/L.

1.5 Statistical analysis

The rhythms of rat pineal melatonin were studied with complex cosine functions (CCF)[21]. Curve fitting was performed using nonlinear regression analysis (Origin 6.1) and parameters for which the significance level of the fitted curve was less than 0.1 were used for further calculating parameter means and examining the rhythm modulation.

2 Results

In this paper, primary rat pineal cell was cultured and the pineal cell culture medium was collected after 3, 6, 9 day culture at 6∶00, 8∶00, 10∶00, 12∶00 and 15∶00 during the day, and at 18∶00, 20∶00, 22∶00, 1∶00 and 4∶00 at night respectively. Each set of data was fitted with the following equation:

$$Y(x) = M + A_{24} * \cos(x - \varphi_{24}) + A_{12} * \cos(2x - \varphi_{12})$$

where x represents the time of day (in radians), Y is the predicted value of melatonin at time x, M is the mesor, A_{24} and φ_{24} is the amplitude and

acrophase of 24 h period respectively, A_{12} and φ_{12} is the amplitude and acrophase of 12 h period respectively. The parameters of those individual showed a significant fit to the CCF ($P<0.1$).

The CCF statistical analysis displayed that the pineal melatonin levels on the third, sixth and ninth days of culture under constant darkness conditions were all matched with a cosine equation (Fig. 1, Fig. 2, Fig. 3). It revealed that the rat pineal cell melatonin synthesis showed an intrinsic rhythm at 3, 6, 9 day successive culture under constant dark conditions. However, the intrinsic rhythm disappeared at 12-day culture. In addition, the results showed that levels of the pineal melatonin synthesis within 24 h slightly increased on the third, sixth, ninth and twelfth day but decreased on the fifteenth day under cell cultured condition(Fig. 4).

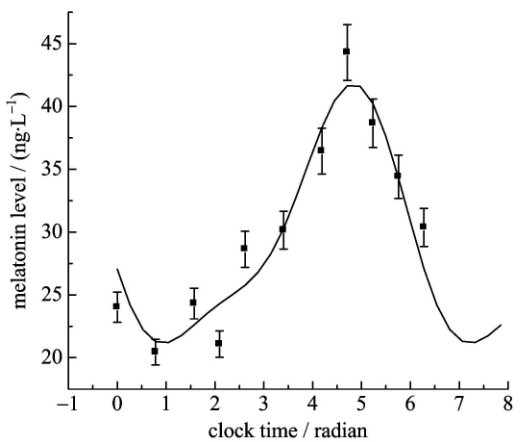


Fig. 1 Mean circadian rhythm of cultured rat pineal melatonin for 3 days under constant darkness conditions and fitted model curve with CCF2

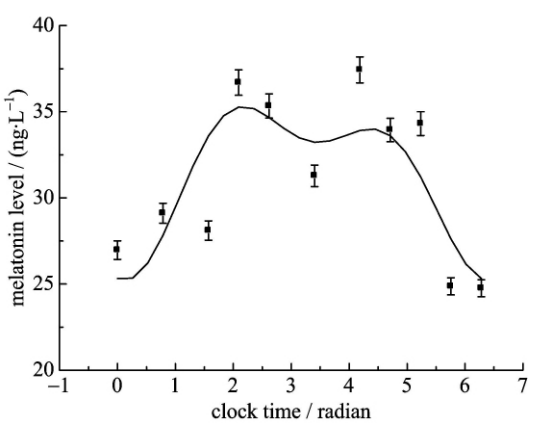


Fig. 2 Mean circadian rhythm of culture rat pineal melatonin for 6 days under constant darkness conditions and fitted model curve with CCF2

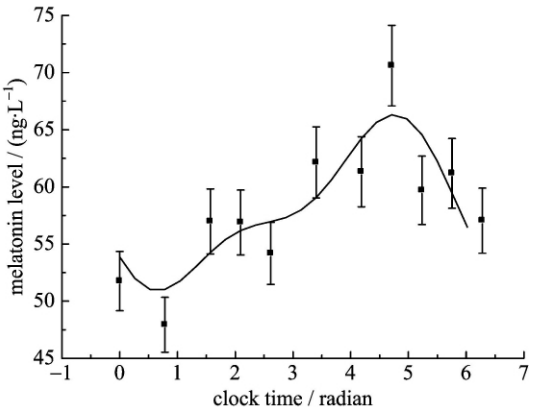
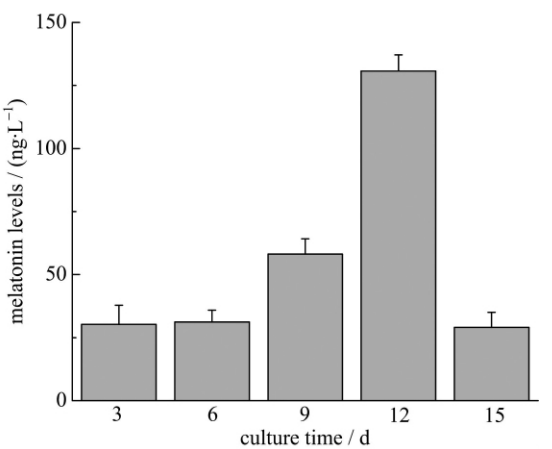


Fig. 3 Mean circadian rhythm of culture rat pineal melatonin for 9 days under constant darkness conditions and fitted model curve with CCF2



Melatonin was determined in the rat pineal cell culture medium. The influence of 10% FBS on melatonin level was eliminated. The cell numbers are all 106.

Values were expressed as mean±SD ($n=11$)

Fig. 4 The mesor of melatonin levels in cultured rat pineal cells under constant darkness conditions

3 Discussion

In this study, cultured individual rat pineal cell is used to test whether melatonin shows intrinsic circadian rhythm under constant darkness after a long time culture. Interestingly, we find that melatonin production from individual rat pineal cells shows a daily fluctuation and its rhythm can be maintained for 9 d under constant darkness conditions. The results in this study are

consistent with those of the previous studies which reported that melatonin rhythm in rat pineal melatonin production persisted under constant darkness (DD) conditions or blinded condition[22]. Previous studies also demonstrated that blinding or exposure to the dark-dark (DD) condition resulted in the induction of the sheep or hamster pineal melatonin circadian rhythm[23-25].

Numerous studies demonstrated that chick pineal melatonin synthesis showing circadian rhythm was regulated by light-dark cycle even in constant darkness which used a flow-through culture system for pineal gland organ culture and found that melatonin release was rhythmical and the melatonin rhythm in individual chicken pineal gland persisted for at least 5 days in vitro[11]. In these studies, the release of melatonin was strongly rhythmic in LD cycles. However, the amplitude of the rhythm was lower and lower in constant darkness.

The mammalian pineal gland has lost photosensitivity during the course of evolution, and information about environmental lighting conditions is imposed on the gland via a complex multi-synaptic pathway[26]. A light signal perceived by the retina is transmitted primarily through the retinohypothalamic tract to the SCN, the site of the master circadian clock. The SCN subsequently conveys the signal to the pineal gland. Whether the pineal gland can receive light signal is unknown. In this study, our CCF statistical analysis revealed that the amplitude of the rat pineal melatonin synthesis at the twelfth day culture was highest and the amplitude of the ninth day culture was higher than that of the sixth and third day culture, respectively. Results are speculated upon that the intrinsic melatonin synthetic rhythms in 3-day-cultured pineal cells could probably be transitional. In addition, the real intrinsic melatonin synthetic rhythms might occur in 9-day-cultured pineal cells and disappear in 12-day-cultured pineal cells. Findings in the present study that the daily fluctuations of melatonin production from cultured individual rat pineal cells persist under constant darkness conditions suggest that rat individual pineal cell perhaps acts as a circadian oscillator.

In conclusion, the present study indicates that melatonin synthesis in 3, 6, 9-day cultured rat pineal cells shows an intrinsic circadian rhythm, and the rhythm mechanism will be further investigated.

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