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Cry1 expression in the chicken pineal gland: Effects of changes in the light/dark conditions

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Abstract

Cryptochromes (Cry) are core components in the gene regulation of circadian rhythmic processes. It was shown earlier, that Cry1 mRNA content of the avian pineal gland was increased after a 4 h exposure to light during subjective night; however, a 30 min exposure was ineffective. In this study, changes in pineal Cry1 expression were detected in chickens during and after being placed into reversed light/dark environment. Cry1 mRNA content was higher if light was on during the night; however, in the first 2 h of light exposure at night, Cry1 mRNA contents were decreased. Following the first overnight light exposure, the peak of the mRNA expression was delayed for 12 h compared to controls. Our results suggest that environmental illumination activates a complex regulatory cascade that includes both up- and down-regulation of the Cry1 gene which inverses the 24 h pattern of Cry1 mRNA expression within one period.

Keywords: Circadian; Avian; Pineal gland; Light; Clock genes; Cry1

1. Introduction

Entrainment of the circadian biological rhythms was shown to be associated with the regulation of clock gene expression by environmental stimuli (Nuesslein-Hildesheim et al., 2000). Cryptochromes (Cry1 and Cry2) are principal mediators of transcriptional regulation in circadian clocks (Kume et al., 1999). Promoter sequences of the Cry1 gene contain binding sites for both repressor (Rev/Erbα, Etchegaray et al., 2003) and activator complexes (Clock/Bmal, Shearman et al., 2000) of the molecular clockwork. Data on the effects of environmental illumination on the expression of Cry1 can provide details on mechanisms which entrain the clock.

Unlike in mammals, the pineal glands of several non-mammalian species, including birds, contain an autonomous circadian clock connected to functioning photoreceptors (Zimmerman and Menaker, 1975; Falcon et al., 1989; Natesan et al., 2002). The avian pineal gland, therefore, can be used well as a model to study how biological clocks are synchronized to the environment.

Although changes in Cry1 expression happen approximately parallel to the phase of the rhythmic environmental illumination (Miyamoto and Sancar, 1999; Yamamoto et al., 2001; Yasuo et al., 2003), a brief exposure to light (15–30 min) during the early phase of the subjective night is insufficient to induce transcription in both avian pineal and mammalian SCN clocks (Miyamoto and Sancar, 1999; Yasuo et al., 2003). These data suggest that Cry1 expression is regulated by a complex light sensitive clock mechanism including a cascade of several steps.

Detecting changes in the rhythm of Cry1 expression during experimental phase-shifts of light/dark cycles is a useful method to collect more data on the mechanisms that synchronize biological clocks (Reddy et al., 2002). Since data on the effects of light on the Cry1 expression of the avian

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pineal oscillator were found controversial (Yamamoto et al., 2001; Fu et al., 2002; Bailey et al., 2003; Yasuo et al., 2003; Helfer et al., 2006), in this study, Cry1 mRNA contents of pineal glands were measured in chickens during and after an overnight light exposure of various durations.

2. Materials and methods

2.1. Animals

Newly hatched white Leghorn chickens were housed under 14/10 h cycles of light/dark environment (light on at 06:00 = ZT 0, i.e., zeitgeber time), at a temperature of 24 °C. The chickens were illuminated with fluorescent lamps providing 400 lux measured at the level of the bird's head. Food and water were available *ad libitum*. Animal housing, care, and application of experimental procedures were in accordance to institutional guidelines under approved protocols (University of Pécs, No. BA02/2000-31/2001).

The experiments were carried out on the 6th week after hatching. In the control groups ($n = 20$ in each experiment), light/dark conditions were not modified. In the first experiment, chickens were exposed to light for 28 h beginning ZT 0 ("exposed" group, $n = 20$). To monitor the acute effects of light at night on the pineal Cry1 expression, pineal glands were collected in 2 h intervals, between ZT 14 and ZT 0.

In the next experiment, light/dark conditions were reversed ("reversed" group, $n = 20$, lights on at the same time as lights off in the control group, i.e. ZT 14). Glands were collected beginning 4 h before the dark phase of the first cycle of reversed light/dark conditions (ZT 0 in the control group). To detect changes in the 24 h pattern of Cry1 expression, taking samples every 4 h provided enough data, as it is commonly accepted. After decapitation, the glands were immediately removed, homogenized, and subsequently frozen at −70 °C.

2.2. Semi-quantitative RT-PCR

Total RNA was extracted from pineal glands with Sigma's TRI Reagent following the manufacturer's protocol. Using 200 ng pineal RNA, one-step RT-PCR was performed with 5 U MMLV Reverse Transcriptase (Applied Biosystems) and 0.2 U RedTaq DNA polymerase (Sigma). After 15 min of incubation at 42 °C and denaturation for 5 min at 94 °C, the reaction was run for 26 cycles (94 °C for 30 s, 58 °C for 30 s, and then at 72 °C for 1 min). The primers for Cry1 mRNA (forward: GAATGCTGGAAGCTGGATGTG, and reverse CCTTCTGGACAC TCTCTGG) were designed earlier in our laboratory (Csernus et al., 2005). To use a 500 bp fragment of the chicken β-actin mRNA for internal standard, GATGGACTCTGGTGATGGTG and AGGGCTGTGATCT CCTTCTG primer pairs were applied. PCR products were separated with 3 mm thin, 2% agarose mini-gels (in TAE buffer), which were post-stained with SYBR Green I (Sigma) and trans-illuminated with blue light (Dark Reader, Clare Chemical Ltd., USA). Pixel intensities of bands on gel photos were measured with Image-J software (NIH). Cry1 expression level was determined by dividing mean band intensities of Cry1 by that of the β-actin.

2.3. Statistical analysis

Differences in Cry1 mRNA contents between samples taken at different time points were analyzed with one-way factorial ANOVA and Fisher's least significant difference (LSD) *post hoc* test. Effect of changes in the environmental illumination on the gene's expression profile was examined with two-way factorial ANOVA. If two-way ANOVA returned significant differences, the difference between experimental and control data at each time point was analyzed with Student's *t*-test. Differences between the effects of a 14 h light exposure at night (first experiment) and the effects of the same exposure after one cycle of reversed light/dark conditions (second experiment) were analyzed with two-way ANOVA, using data collected at ZT 16 and ZT 20.

3. Results

3.1. Changes in Cry1 mRNA contents of chicken pineal glands during 14 h of light exposure at night

Pineal samples were collected during the subjective night period (between ZT 14 and ZT 0), in 2 h intervals. During exposure, pineal Cry1 expression decreased in 2 h ($p = 0.040$, Fig. 1). In turn, 8 h after the beginning of the exposure, Cry1 mRNA contents were increased ($p = 0.050$ at ZT 22 and $p = 0.042$ at ZT 0). In control chickens, Cry1 mRNA expression decreased during the night ($p = 0.029$).

Light exposure altered the pattern of Cry1 expression at night ($p = 0.0037$). Compared to control, after the beginning of the exposure, Cry1 mRNA contents were lower in 2 h ($p = 0.0433$), but higher in 8 h ($p = 0.0429$ at ZT 22 and $p = 0.0234$ at ZT 0, Fig. 1).

3.2. Effects of a reversed light/dark cycle on the 24 h pattern of Cry1 expression in the chicken pineal gland

Pineal glands were collected between ZT 0 (4 h before lights off in the reversed group) and ZT 20 in 4 h intervals. Pineal Cry1 expression showed episodic changes already in the first cycle of reversed light/dark conditions ($p = 0.000074$, Fig. 2). mRNA contents decreased 4 h after lights off (ZT 8, $p = 0.0083$ or $p = 0.0487$ if related to ZT 0 or ZT 4, respectively). By the end of the first reversed cycle, Cry1 expression increased (ZT 12, $p = 0.00097$), and remained at the same level during the first 6 h of the next cycle ($p = 0.0364$ and $p = 0.0252$ at ZT 16 and 20, respectively). In control chickens, changes in Cry1 mRNA

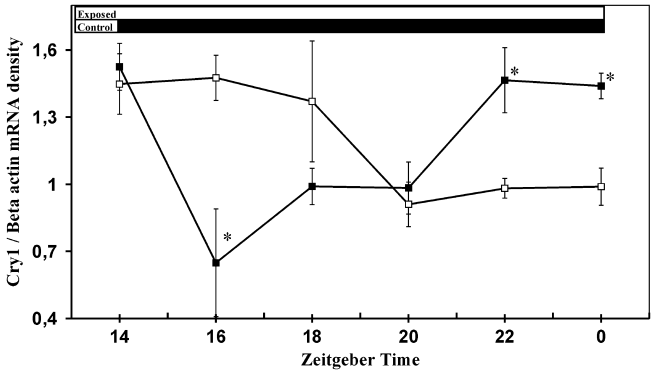

Fig. 1. Changes in Cry1 mRNA contents of chicken pineal glands during 14 h of light exposure during subjective night. Graphs represent means ± SEM of Cry1/β-actin mRNA contents of 2–4 glands (filled squares, experimental group; empty squares, control group). Horizontal bars show light/dark conditions for each group (black indicates dark period). Significant differences ($p < 0.050$) between experimental and control groups are shown with asterisks. Results of statistical analysis (*p* values): Exposed group: ANOVA, 0,019; *post hoc* ZT14, 0.04; ZT18, 0.155; ZT20, 0.168; ZT22, 0.050; ZT0, 0.043. Control group: ANOVA, 0.083; *post hoc* ZT14, 0.043; ZT16, 0.029; ZT18, 0.126; ZT22, 0.29; ZT0, 0.302. Comparison of groups: ANOVA: exposure, 0.803; Zeitgeber, 0.038; interaction, 0.0037. Student's *t*-test: ZT14, 0.348; ZT16, 0.043; ZT18, 0.155; ZT20, 0.340; ZT22, 0.043; ZT0, 0.023.