Available online at www.sciencedirect.com
ScienceDirect
Comparative Biochemistry and Physiology, Part A 149 (2008) 44 – 50
CBP
www.elsevier.com/locate/cbpa

Ontogeny of melatonin, *Per2* and *E4bp4* light responsiveness in the chicken embryonic pineal gland

I. Herichová [a], J. Monošíková [a], M. Zeman [a,b,*]

[a] *Department of Animal Physiology and Ethology, Comenius University Bratislava, Mlynská dolina B2, 842 15 Bratislava, Slovakia*
[b] *Institute of Animal Biochemistry and Genetics, SASci, 900 28 Ivanka pri Dunaji, Slovakia*

Received 17 July 2007; received in revised form 2 October 2007; accepted 2 October 2007
Available online 11 October 2007

Abstract

The chicken pineal gland possesses the capacity to generate circadian oscillations, is able to synchronize to external light:dark cycles and can generate an hormonal output — melatonin. We examined the light responses of the chicken pineal gland and its effects on melatonin and *Per2*, *Bmal1* and *E4bp4* expression in 19-day old embryos and hatchlings during the dark phase, subjective light phase and in constant darkness. Expression of *Per2* and *E4bp4* were rhythmic under light:dark conditions, but the rhythms of *E4bp4* and *Bmal1* mRNA did not persist in constant darkness in 19-day old embryos. *Per2* mRNA expression persisted in constant darkness, but with a reduced amplitude. *Per2* expression was inducible by light only during the subjective day. Melatonin release was inhibited by light only at end of the dark phase and during the subjective light phase in embryos. Our data demonstrate that the embryonic avian pineal pacemaker is light sensitive and can generate rhythmic output, however the effects of light were diminished in chick embryos in compared to hatchlings.

Keywords: *Bmal1*; Circadian; Entrainment; Phase dependence; Pacemaker; Clock

1. Introduction

Biological rhythms in physiological functions are driven by the circadian system and synchronized to external cycles via several sensory structures. In birds the central part of the circadian system is composed of the pineal gland, the retina and the suprachiasmatic nucleus of the hypothalamus (SCN) (Cassone and Menaker, 1984). Unlike in mammals, the avian pineal gland is an autonomous circadian oscillator, is directly photosensitive and generates a rhythmic melatonin output signal (Takahashi et al., 1989; Zatz et al., 2000). Melatonin plays an important role as a signal mediating information about external photic conditions to the rest of the body (Gwinner and Brandstätter, 2001).

In our previous work we reported that melatonin synthesis by the pineal gland is rhythmic on embryonic day (ED) 19 and is entrainable by light:dark (LD) or temperature cycles (Zeman et al., 1999, 2004). Expression of the rate limiting enzyme for melatonin synthesis, arylalkylamine *N*-acetyltransferase, is also rhythmic in the pineal gland on ED 19 (Herichova et al., 2001). Embryonic development of the avian pineal gland function was further demonstrated by *in vitro* studies (Lamosova et al., 1995; Csernus et al., 2007).

Molecular circadian oscillations are generated by a complex set of feedback loops formed by clock gene transcripts and their protein products (Shearman et al., 2000). In birds, homologous clock genes were cloned (for rev. see Fukada and Okano, 2002) and their functions seem to be similar to those in mammals (Okano et al., 2001).

Chicken *Per2* mRNA is expressed rhythmically in the pineal gland on ED 18 when embryos are exposed to a LD cycle (Okabayashi et al., 2003), but levels of *Per2* mRNA were low and arrhythmic when embryos were incubated in constant darkness. These findings raise the question as to whether the pineal circadian oscillator needs to be stimulated by a synchronizing

* Corresponding author. Department of Animal Physiology and Ethology, Comenius University Bratislava, Mlynská dolina B-2, 842 15 Bratislava, Slovakia. Tel.: +421 2 602 96 424; fax: +421 2 654 29 064.
E-mail address: mzeman@fns.uniba.sk (M. Zeman).

1095-6433/$ - see front matter © 2007 Elsevier Inc. All rights reserved.
doi:10.1016/j.cbpa.2007.10.006

signal during embryonic development in order to become functional.

Previously we showed that a single LD cycle during the first day post-hatching is sufficient to entrain the avian circadian system in the chicken, regardless of whether the eggs were previously incubated under LD or constant darkness (DD) (Zeman et al., 1999). This finding suggests that a functional embryonic pacemaker can develop in DD. The molecular circadian oscillator and melatonin synthetic pathways are interconnected in avian pinealocytes (Chong et al., 2000; Okano et al., 2001; Rekasi et al., 2006), therefore the machinery for the molecular pacemaker should develop before or, at a minimum, at the same time as rhythmic melatonin synthesis in the pineal gland.

To elucidate correlations between rhythmic melatonin synthesis and clock gene expression in the pineal gland we designed several experiments focusing on the ontogeny of light responsiveness by the pineal gland. We examined the sensitivity of the melatonin signal to light pulses during the subjective night and day and compared these responses with the expression of photoinducible clock genes. To understand the developmental state of the circadian pacemaker, we analyzed the expression of the photoinducible genes *Per2*, *Bmal1* and *E4bp4* in the pineal gland of chick embryo prior to hatching and immediately post-hatching under LD and DD conditions.

2. Materials and methods

Hatching eggs of broiler breeders (*Gallus gallus domesticus*) were incubated in forced draught incubators (BIOS Midi, Sedlčany, Czech Republic) at a temperature of 37.3 ± 0.3 °C and relative humidity 55–65%. Eggs were turned automatically every two hours. Lighting was provided by an 18 Watt cool white fluorescent tube (Osram, Lumilux combi, Germany), with illumination ranging from 40–80 lux at the level of the eggs. The light:dark cycle, light pulses and sampling times are given separately for each particular experiment. Two identical forced draught incubators (BIOS Midi, Sedlčany, Czech Republic) were used in experiments involving different lighting conditions and light pulses. During the dark-phase, eggs and chicks were taken from the incubator in complete darkness and decapitation occurred within 10 s in the neighboring room under very low intensity red light (15 W, Kodak 1A filter). After decapitation pineal glands were excised, frozen in liquid nitrogen and stored at −80 °C until melatonin measurement or RNA extraction was performed. Experimental protocols were approved by the Ethical Committee for the Care and Use of Laboratory Animals at the Comenius University Bratislava, Slovak Republic.

2.1. Experiment 1: Effects of a two hour light pulse on melatonin synthesis in the pineal glands of 19-day old chick embryos

Incubated eggs (75) were exposed to a 16:8 LD cycle. Sampling was performed in 2–3 h intervals on day 19 of embryonic development. A single two hour light pulse was applied during the dark period at intervals ZT16–ZT18, ZT18–ZT20, ZT20–ZT22 or ZT22–ZT24 (ZT — Zeitgeber time, the onset of the light phase is considered ZT0). Control eggs were incubated under the original LD regimen (16:8) and maintained in darkness during the light pulse treatment.

2.2. Experiment 2: Effects of a three hour light pulse on pineal melatonin synthesis in the pineal glands of 19-day old chick embryos and hatchlings

Hatching eggs (51) were incubated under a 12:12 LD cycle. On ED 19 embryos were exposed to constant darkness and a single three hour light pulse was applied at intervals ZT18–ZT21, ZT21–ZT24, ZT3–ZT6 or ZT6–ZT9. Control eggs were incubated under the original LD regimen and during the light pulse treatment were maintained in the 12:12 photoperiod.

2.3. Experiment 3: Daily rhythms of Per2, Bmal1 and E4bp4 expression in the pineal glands of 4-day old chicks synchronized to a LD cycle

Eggs (24) were incubated under a 12:12 LD cycle until hatching. After hatching, chicks were kept in a brooder room with the same LD cycle as in the incubator. The ambient temperature in the room was kept constant at 37 °C for the first 2 days and 34 °C for the next 2 days. Food and water were available *ad libitum*. Samples were taken over a 24 h cycle in 4 h intervals with the first sampling time at ZT14.

2.4. Experiment 4: Daily rhythms of Per2, Bmal1 and E4bp4 expression in the pineal glands of 19-day old chick embryos synchronized to a LD cycle or in DD

Eggs (54) were incubated under a 12:12 LD cycle. On ED 19, pineal glands were taken at 4 h intervals during the scotophase, with the first sampling at ZT 14. At the end of the scotophase (ZT24) 18 embryos were exposed to the regular light phase and 18 embryos were incubated in constant darkness. Pineal glands from both groups were taken at ZT2, ZT6 and ZT10.

2.5. Experiment 5: Effects of 1 h or 3 h light pulses on clock gene expression in the pineal glands of 19-day old chick embryos

Eggs (42) were incubated under a 12:12 LD cycle and on ED 19 were exposed to constant darkness. Three hour light pulses were applied in the middle of the subjective day (ZT3–ZT6) or middle of the subjective night (ZT15–ZT18). One hour pulses were performed at intervals ZT17–ZT18 or ZT5–ZT6. Samples were taken at the end of each light pulse. Control eggs were incubated under the original LD regimen 12:12 in another incubator during the light treatment. Control groups were sampled in the middle of the dark phase or in the middle of the light phase.

2.6. Experiment 6: Effects of a 1 h or 3 h light pulse on clock gene expression in the pineal glands of 4-day old chicks

Eggs (32) were incubated in a 12:12 LD cycle until hatching. After hatching, chicks were kept in a brooder room under the