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The embryonic pineal gland of the chicken as a model for experimental jet lag

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ABSTRACT

The circadian clock in the chicken pineal model develops before hatching, at around the 17th embryonic day (ED17). By this stage, it runs in synchrony with environmental cues. To address if phase resetting mechanisms are comparable to those of post-hatched chicken, we investigated ED19 stage chicken embryos under 12 h light:12 h dark (LD), under constant darkness (DD), or under acute 4 h phase delay of the LD condition (LD + 4). The 24 h mRNA-expression patterns of clock gene *clock* and of clock controlled genes *Aanat* and *hiomt* were analyzed with qRT-PCR. Under DD the rhythm of *Aanat* did not change significantly, however the 24 h pattern of *hiomt* was altered. *Clock* shows a delayed response to DD with a phase-shift in its rhythm. After the first cycle under LD + 4 conditions, the 24 h patterns of *Aa-nat* and *hiomt* mRNA-s were phase delayed. *Clock* showed both acute and delayed changes in response to LD + 4. These results show that the embryonic chicken pineal gland has a fully functioning clock mechanism, and that it is a good model for phase-change experiments. In addition it demonstrates that only one cycle of altered light schedule is sufficient to trigger changes within the molecular clock mechanisms of the chicken embryonic pineal model.

1. Introduction

Changes in the light schedule is known to cause changes within circadian clock mechanisms which is seen e.g. in jet lag after transatlantic flights. Such changes may also lead to health problems which have been extensively documented in shift workers who demonstrate amongst others an increased risk of metabolic syndrome, breast cancer, colon cancer and cardiovascular diseases (Rajaratnam and Arendt, 2001; Davis, 2006; Boivin, 2007; Karlsson, 2001; Eckel-Mahan, 2013; Rohling, 2011). Light is known to be one of the main entraining factor for the circadian rhythm across species like rats, mice, flies, birds and even neurosporra crassa (Rohling, 2011; Chen Rongmin, 2008; Stehle, 2003; Korf, 2003; Bell-Pedersen, 2005). The effect of light on the circadian clock depends on what time of day it is given. Light during early subjective night causes phase delay, light at late subjective night causes phase advance whereas light during subjective day has little or no effect on the circadian clock (Korf, 2003; Dijk Derk-Jan, 2012; Hirota et al., 2011). This can be seen both at the level of transcriptional activity of circadian clock genes, as well as at the level of melatonin release from the pineal gland (Cassone, 1990). Experiments which have been done on mice have demonstrated that the circadian system responds faster to a phase delay of the rhythm rather than a phase advance Pfeffer et al. postulated that this in part may be due to the fact that lights left on for longer, which occurs in phase delay experiments, will typically inhibit locomotor activity in mice, thus the speed at which a change in locomotion is seen in response to phase delay may be directly due to the lights being left on.

Melatonin is a key hormone in the circadian system; it works as a photoperiodic signal giving the organism information about the day/night cycle (Bailey et al., 2009). In mice melatonin has also been shown to be involved in phase-resetting of the circadian rhythm in response to changes in the light schedule (Pfeffer, 2012) The avian pineal gland can generate rhythmic expression of melatonin both under *in vivo* and *in vitro* conditions (Cassone, 1990). In birds the production of melatonin can be detected already from the second week of embryonic development during *in vitro* conditions (Zeman, 2011). Then the amplitude rhythm of melatonin expression increases dramatically during the last two days of development reaching a peak at the embryonic day 20 (ED20), seen as an increase in the levels *AANAT* (arylalkylamine-*N*-acetyltransferase) protein concentration within the pineal gland at ED20 (Zeman, 2011). In birds the production of melatonin in the pineal gland directly corresponds to the changes in mRNA expression of *AANAT*, therefore measuring the mRNA of *AANAT* is sufficient to study the rhythmic production of melatonin in this model (Zeman, 2011; Zatz et al., 2000; Klein, 2006)

CLOCK is a circadian clock protein acting together with *Bmal1* and *Npas2* as a positive transcriptional regulator. Knockdown of *Clock* expression has been shown to cause a decrease of *Aanat* activity, as well as a decrease of *Npas2* and *Per2* demonstrating that this is an essential component of the circadian clock (Haque, 2010). Amongst its functions it forms a heterodimer with *Bmal1* to induce

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the expression of *AANAT* (Haque, 2010). This feature of *Clock* has been demonstrated during development of fish (Martin-Robes, 2010) and chicken (Goncalves, 2012).

The study of the circadian clock under development has been done in a variety of species. The embryonic chicken pineal gland has been shown to have a working clock mechanism which is synchronized to environmental stimuli by ED 17 (Nagy, 2008). Martin-Robes et al. reported the presences of clock genes whose rhythm is responsive to changes in light already from the 1st day in the developing Senegalese sole. In rats *hiomt Aanat* and *melatonin* have been shown to be rhythmic already from an early stage of development (Jimenez-Jorge, 2007), and the rhythm of these are also light responsive. In zebra fish studies have shown that already 22 h after fertilization the master pacemaker, the pineal gland, is developed, and already 2 days after fertilization melatonin is rhythmic in expression as long as the fish are exposed to light and dark (Smadja Storz et al., 2013). Earlier it has been demonstrated that the mRNA expression of *clock* responds rapidly to acute phase delay in the adult chicken pineal glands (Kommedal et al., 2011). However as of yet the effect of phase advance on the expression of *clock* and clock controlled genes within the embryonic chicken pineal glands have not been studied.

To demonstrate that the embryonic chicken pineal gland has a fully functioning clock mechanism which is comparable to that of adult chicken we have looked at the expression of *clock* and the genes involved in melatonin synthesis, i.e. *Aanat* and *Hiomt* under 12 h:12 h light:dark (LD) and constant dark (DD) *in vivo* conditions. Furthermore, we have examined the effect of a four hour phase delay (LD + 4) on the expression of these genes in the embryonic chicken pineal gland *in vivo*.

2. Materials and methods

2.1. Animals

Fertilized eggs of white Leghorn chicken were incubated from embryonic day 1 (ED1) at a temperature of 37.5 °C, and humidity between 60% and 70%. Egg rocking occurred automatically every 2 h. Control eggs were kept under 12 h light 12 h dark, LD conditions. Experimental groups were from ED19 either placed under DD or subject to a shift of the LD cycle by four hours effectively delaying the lights off time point by 4 h (LD + 4). The chicken embryos were sacrificed by decapitation every four hours from ZT 12 (time of lights off in the control group) between ED19 and ED 21 and the pineal glands were collected.

2.2. Real-time quantitative RT-PCR

Total RNA was extracted from pineal glands with Sigma's TRI Reagent following the manufacturer's protocol (T9424, Sigma–Aldrich, St. Louis, Missouri, USA). cDNA was generated by reverse transcription using 500 ng of total RNA in a total of 50 μL reaction volumes following the instructions of the manufacturer (Applied Biosystems, SuperScript RT enzyme, 4374966). Real-time PCR was run using 3 μL cDNA solution per 15 μL reaction volume with Applied Biosystems' TaqMan gene expression assays for *Aanat*, *hiomt* and *clock* and also for internal reference gene β-*actin*.

Composition of reaction mixture was made based on the instructions of the manufacturer (Applied Biosystems, 4370048). StepOne Real-Time PCR instrument (Applied Biosystems) was used with default cycling parameters (50 °C 2 min, 95 °C 10 min, 40 cycles of 95 °C 15 s and 60 °C 1 min). For quantitation calculations the delta–delta Ct method was used, after control measurements for reaction efficiency normalizations.

2.3. Data analysis

Group differences were evaluated using two-way ANOVA followed by Tukey's post hoc test. $P < 0.05$ was considered as statistically significant difference.

3. Results

3.1. In vivo effect of constant darkness (DD) compared to normal LD on the expression of Aanat in the embryonic chicken pineal gland

On ED 19 the eggs were placed under DD. Glands were collected starting from zeitgeber time (ZT) 12, 12 h prior to the switch in the lighting conditions, every four hours until ED 21. The control group was kept under LD conditions. *Aanat* showed a robust daily rhythm, even under DD conditions (Fig. 1A). Lack of light at ZT 4:00 does not cause a significant change in the expression of *Aanat*. In fact there is no significant difference in the expression of *Aanat* between the LD and DD groups throughout the duration of the experiment.

3.2. In vivo effect of constant darkness (DD) compared to normal LD on the expression of Hiomt in the embryonic chicken pineal gland

Eggs of ED19 chicken were placed under DD. Pineal glands were collected every four hours until ED21. For the LD group *Hiomt* has a high amplitude rhythm with a peak during the light phase at ZT4 (Fig. 1B). Under DD conditions at ZT4 the absence of light causes a shift in the expression of *Hiomt* compared to that seen under LD (Fig. 1B). Then in the second cycle of DD there is a lower expression of *Hiomt* at ZT4, compared that seen under LD conditions.

3.3. In vivo effect of constant darkness (DD) compared to normal LD on the expression of clock in the embryonic chicken pineal gland

The eggs were placed under DD at ED 19, and the pineal glands were collected every four hours until ED21. In the control group, which is kept under normal LD conditions, *clock* mRNA shows a peak during the light phase (Fig. 1C). In the DD group at ZT 4, four hours after a change in the light schedule there was no significant difference in the level of *clock* mRNA compared to the LD group. Then at ZT8 there is an increase in the expression of *clock* mRNA compared to that seen under LD conditions. In the second cycle of DD the expression of *clock* mRNA is suppressed (Fig. 1C).

3.4. In vivo effect of four hour phase delay on the expression of Aanat in the embryonic chicken pineal gland compared to normal LD conditions

On ED19 the light schedule was changed in such a way that lights were left on for 4 h longer (LD + 4) than in the control LD group, effectively causing a four hour phase delay. Pineal glands were collected every four hours starting from ZT12 which is prior to the phase delay. Samples were collected over a 48 h period. In the LD group *Aanat* shows a clear robust 24 h rhythm (Fig. 2A). In the LD + 4 group during the first four hours of phase delay, at ZT16, there was no acute change in the expression of *Aanat* when compared to the LD group. However, looking at the second cycle of LD + 4 conditions a clear phase delay of the expression of *Aanat* is observed.