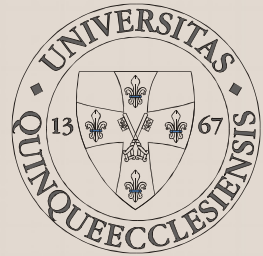


PH. D. THESIS (SUMMARY)

# **CLOCK GENE EXPRESSION STUDIES ON THE CHICK PINEAL CLOCK MODEL**

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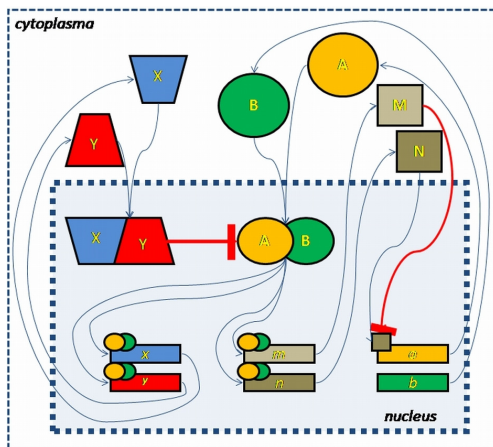
Advisor: Dr. Valér Csernus

Pécs, 2010.

## INTRODUCTION

CIRCADIAN BIOLOGICAL RHYTHMS. There is an increasing need for knowledge about endogenous timing mechanisms affecting the boundaries of human performance: health and disease. Biological rhythms are phenomena recurring in a living organism with a typical period length by which periodical changes in the environment (light, temperature, food availability, stress, *etc.*) can be anticipated. *Circadian* biological rhythms show a temporal pattern which tracks light/dark cycles resulting from Earth's rotation around its own axis (*circa*- approximately, *diem*- a 24 h day). Circadian rhythms are ubiquitous in biology showing the following rules: (1) it is endogenous: the rhythm still keeps running with a period length close to but never exactly 24 h in an artificially nonperiodic environment of *constant* darkness, temperature and humidity (referred as DD). (2) Within one period, the actual stage (phase) of the rhythm can be easily shifted with a proper environmental stimulus (*zeitgeber*: german „time giver”).

THE CIRCADIAN CLOCK. Biological mechanisms driving circadian rhythms were referred to *biological clocks* from the 1960's. *Circadian clocks* work with remarkable precision, having all the components of the pacemaking oscillator within a cell. Repetition is controlled in *eukaryotes* at the transcriptional level with negative feed-back. It became clear only in the 1990's that the feed-back loop consists of only two complexes made up of activator or repressor clock proteins encoded by the *clock genes*. CLOCK/BMAL heterodimers enter the nucleus and bind to *E-box* promoter elements, thereby inducing



transcription. E-boxes are found in both *per* genes and *cry* genes, of which the protein products form heterodimers interacting with the CLOCK/BMAL complex in an inhibitory way. The inhibition is turned off by degradation of clock proteins which allows the initiation of a new cycle. This involves a secondary feed-back loop regulated by REV-ERB $\alpha$  and ROR $\alpha$  proteins (**Figure 1**).

**Figure 1.** The molecular model of the circadian biological oscillator within a cell. Molecular interactions are discussed in the text. Key clock genes in vertebrates (rodents): a: *bmal*, b: *clock*, x: *per*, y: *cry*, m: *Rev-erb $\alpha$* , n: *Ror $\alpha$* .

THE ROLE OF THE CIRCADIAN CLOCK IN PHYSIOLOGY. The timer function of circadian oscillators is used not only by homeostatic regulatory systems, but also by the cardiovascular-, endocrine- and the immune system. Circadian rhythm is presented in a broad spectrum of physiological parameters as daily fluctuations with an average of 20-30% changes in the amplitude. Up to 90% of cell-specific genes may show 24 h rhythmic pattern in their expression. Furthermore, fundamental cellular processes (*e.g.* cell cycle) are under the control of the molecular oscillator, suggesting a more general

„housekeeping” role for the circadian clock in biology. Its involvement in the development and outcome of several endemic diseases has already been proven.

REGULATION OF THE CIRCADIAN CLOCK IN MAMMALS. The most obvious environmental stimulus in this process is the alteration of light/dark cycles. For decoding, *intrinsically photosensitive retinal ganglion cells (ipRGC)* play a key role, which project to non-image-forming brain areas. The *retinohypothalamic tract (RHT)* ends on the *suprachiasmatic nucleus (SCN)*, the ablation of which results in disruption of rhythmicity in key physiological functions. In mammals, the SCN is the primary pacemaker for circadian rhythms. Unlike other cellular oscillators (*e.g. fibroblast, hepatocyte*), SCN neurons oscillate and keep being synchronised to each other even in the absence of any *zeitgeber*, due to abundant intercellular connections. Light exposure at night induces in *retinorecipient* SCN neurons the expression of *c-fos*, *c-jun* and *per* genes resulting in phase-shift of all SCN-driven rhythms in physiological processes. The phase-dependency of this effect is controlled at the level of signalling. In the control of the light evoked signaltransduction of the SCN (Glu – NMDA-R -  $\text{Ca}^{2+}_i$  - CaMK IV – b-Raf – MEK1 – ERK1 – MSK – CREB – CRE – *per*), PACAP plays a key role as a neuromodulator released from the RHT. After adjusted to the environmental light/dark cycle, the phase-signal is transmitted by SCN neurons to nearby hypothalamic centres (homeostats: body temperature, energy balance, sleep/wake, locomotor activity/inactivity; autonomic nervous system; *hypothalamo-hypophyseal* system). A characteristic phase-delay is seen between clock gene expression rhythms in SCN neurons *vs.* SCN-driven peripheral oscillators which is caused by tissue specific differences in receptors and signalling pathways. Among the SCN-driven endocrine organs, the pineal gland has a special role in the systemic control of circadian rhythms: (1) it serves with a special circulating *zeitgeber* signal (*melatonin*, „darkness hormone”) and (2) it is needed in the regulation of photoperiod dependent biological processes (*e.g.* seasonal breeders).

THE AVIAN PINEAL GLAND, AS A CIRCADIAN CLOCK MODEL. Although the 24 h rhythm of melatonin-secretion is a phylogenetically well conserved function, the *pineal body* shows significant species-specific morphological and functional differences. The avian pineal gland seems to be particularly useful for modelling circadian regulatory mechanisms, since it shows all the advantageous features listed below: (1) It is localised in the close proximity of the calvaria, where the bone is thin and translucent. (2) The presence of both cone-like *photoreceptor cells* and *pinealocytes* indicates a phylogenetic change in the differentiation of pineal progenitor cells, in which the appearance of *pinealopetal* nerve fibres play a key role during ontogenesis. (3) The amplitude of melatonin-release can be influenced *in vitro* both by direct light exposure and by adrenergic ( $\alpha_2$ ) stimuli. (4) Unlike the pineal gland of mammals and other non-mammalian vertebrates, the avian pineal gland oscillates *in vitro* under DD conditions for >5 cycles, which makes it comparable with the mammalian SCN oscillator. (5) *Pinealectomy* in birds, but not in mammals may result in disruption of many circadian rhythms. (6) In contrast to the mammalian SCN, the avian pineal gland provides an *in vitro* model with both intact histomorphological structure and a complete isolation from the neuroendocrine/paracrine effects of nearby tissues. (7) The pineal gland of chicken can be obtained as a whole and can be subjected

for *in vitro* experimentation from as early as pre-hatch (embryonic) stages, thus making it an ideal model for studying environmental and genetical factors which are needed to develop and synchronise circadian rhythms independently from the maternal humoral environment.

By the late 1990's the mammalian SCN model has become accessible with a broad spectrum of genetical and molecular biological methods. In 1999, as I joined the student research group of the Anatomy Department (Medical School, University of Pécs), our aim was to proof the old hypothesis at the level of clock gene expression, *i.e.* the chicken pineal model is comparable to the mammalian SCN model with some features being more advantageous for the description of basic mechanisms of circadian rhythmic regulatory processes.

## AIMS

- (1) At the beginning of our work we aimed to identify the clock genes of chicken.
- (2) To determine the 24 h pattern of clock gene mRNA expression in the chicken pineal gland, we planned to study chickens kept under normal light/dark (LD) conditions.
- (3) We planned *in vivo* and *in vitro* experiments in order to collect data about the role of pineal photoreceptors of the chicken in the control of clock gene expression by comparing the effects of different light/dark cycles.
- (4) The circadian rhythm of melatonin synthesis in the chicken pineal gland is controlled at the transcriptional level from the 17<sup>th</sup> embryonic day under LD conditions. To clarify if this developmental event was related to synchronised oscillation of the molecular clock, we compared the 24 h pattern of pineal clock gene mRNA expression between chicken embryos incubated under LD and 6 weeks old chickens kept under LD cycles.
- (5) Cellular oscillators may work in the chicken pineal gland already before hatching; however, it was not clear if environmental stimuli would be necessary to establish synchrony. To clarify this at the level of clock gene expression, we planned *in vivo* and *in vitro* experiments on chicken embryos incubated under constant darkness (DD).
- (6) It was speculated earlier that like in the case of the mammalian SCN, also avian pineal oscillators may use PACAP to establish synchrony. However, data collected before did not support this idea. In our study we wanted to answer the question, if a similarly timed and dosed *in vitro* PACAP treatment, which is comparable to what was reported in mammals to be effective, would have any effect on the 24 h pattern of clock gene mRNA expression in the pineal gland of chicken embryos incubated under DD conditions.

## MATERIALS AND METHODS

**OLIGONUCLEOTIDE PCR PRIMERS.** At the beginning of our work there was no clock gene sequence known in birds, yet. Our attention was caught by the gene of the photosensitive clock protein CRY1: we were searching for homologue sequences based on the mouse *cry1* mRNA in a chick EST library (<http://www.chick.manchester.ac.uk>). Later, we used avian sequences for the examination of *per1*, *per2*, *per3*, *cry2*, *clock*, *bmal1* and *bmal2* clock genes, as these could be attained from GeneBank only later (**Table 1**). The oligonucleotide primerpairs were designed to overlap intronic sequences in the mouse genes. Furthermore, the size of RT-PCR products were set to 100-500 bp, and the size of genomic PCR products at least 100 bp longer. *Cry1* specific RT-PCR products were sequenced in the Pathology Department in 2001, while *cry2* and *clock* RT-PCR products were sequenced in the Laboratory Medicine Department in 2008.

Clock genes	GeneBank ID codes	Species	forward primer sequence	Reverse primer sequence	Predicted product size	Intron size
<i>clock</i>	AF 246959	chicken	tcccagctctctggacaacc	gctgttgctggatcatgtgt	420	800
<i>bmal1</i>	AF 246957	chicken	tggcagaccaaaggatgg	ttcagacatagcgttgcaac	370	700
<i>bmal2</i>	AF 246958	chicken	atgcacaacaaccgaacatc	caatgttgctccatttgaage	330	900
<i>per1</i>	NM 011065	mouse	cggagcttctgggttgc	cccaggggatgggactc	275	1200
<i>per2</i>	AF 246956	chicken	caatggctcacacgaacact	ggagcagctgtagctgtggt	250	1050
<i>per3</i>	AY 046567	chicken	cacaggttcagcagcatcag	atcatcaccaggctgactc	225	1300
<i>cry1</i>	AY 034432	chicken	gaatgctggaagctggatgtg	ccttctggacactctctgg	200	100
<i>cry2</i>	AY 034433	chicken	acaagccaccctcacctac	gatgggaccccatcacatc	150	700

**Table 1.** PCR primers for the detection of chicken clock gene mRNA and genomic DNA.

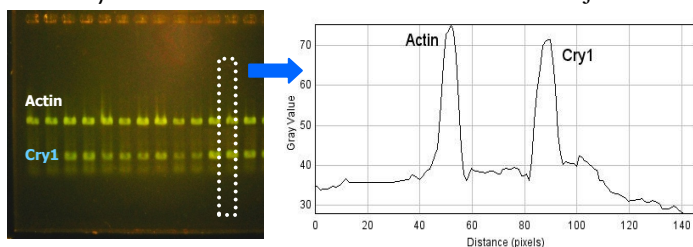
Those primer pairs were selected which would amplify RNA and genomic DNA fragments with the sizes (basepairs, bp) indicated above.

**REAGENTS.** For total RNA extraction of tissue specimens, TRI reagent was used (Sigma T9424). Applied Biosystems' MuLV reverse-transcriptase (N8080018) was used in the RT reaction and Sigma's RedTaq (D4309) for the PCR. The agarose gel-electrophoresis was run in TAE buffer. Ethidium-bromide (Sigma E1385) or SYBR Green I (Sigma S9430) was used for nucleic acid staining.

**ANIMALS, TISSUE SAMPLING.** Chicken mRNA and genomic DNA samples were obtained from pineal glands of white *Leghorn* chickens (Pécs-Reménypusztá Poultry Processing Ltd.). All efforts were made to minimize animal suffering. Chickens were decapitated right before collecting pineal samples.

**SEMIQUANTITATIVE RT-PCR.** To normalize the efficiency of RT reactions and the differences in initial RNA contents, an internal standard of  $\beta$ -actin gene was used. One-

step RT-PCR protocol was optimized for the processing of pineal RNA samples. The reaction parameters were as follows: first strand synthesis 42°C 15 min, denaturation 94°C 5 min, amplification for 26 cycles (94°C 30 s, 60°C 30 s, 72°C 1 min), and final extension of 72°C 7 min. The protocol was set for *clock*, *cry1* and *cry2* primers with 1,5 mM of Mg<sup>2+</sup> concentrations. RT-PCR products were separated with gel-electrophoresis of 5 µl of the reaction mixture. The agarose gel was stained with *SYBR Green* followed by transillumination with blue light (Dark Reader, *Clare Chemical*, USA). For the detection of emitted fluorescent signals we constructed a gel documentation system (tent, *Canon A510* LCD camera, *amber filter* - *Clare Chemical*). *SYBR Green* fluorescence of PCR products were determined from the mean of background-subtracted pixel intensities of DNA bands (**Figure 2**). Fluorescence of *clock* gene RT-PCR products were normalized to those of *β-actin*: the obtained relative values were subjected to statistical analysis.



**Figure 2.** Semi-quantitative analysis of *cry1* RT-PCR products. The *SYBR* stained agarose gel shows *actin* and *cry1* fragments amplified from chicken pineal RNA samples. The histogram shows pixel intensity of the dotted lane on the gel-photo.

To approximate changes in mRNA expression, standard curves for RT-PCR products were obtained. Data for fold changes in initial template amounts were calculated from extrapolating raw fluorescence of products of 2-fold serial dilutions of cDNA and RNA within the exponential range. A two-fold increase in relative *SYBR Green* fluorescence of PCR products correlated with at least a 1.4-fold (*clock*) or 2.4-fold (*cry1*) increase in initial amounts of cDNA transcripts. Two-fold increase in *SYBR Green* fluorescence of RT-PCR products correlated with at least 2.2-fold (*clock*) or 3-fold (*cry1*) increase in initial amounts of total RNA. *SYBR Green* fluorescence of experimental samples were adjusted to cDNA standards and also corrected with the size differences of fragments. As a result, transformed data were plotted on charts to show the mean approximated values of initial *clock* gene mRNA contents in each experimental group.

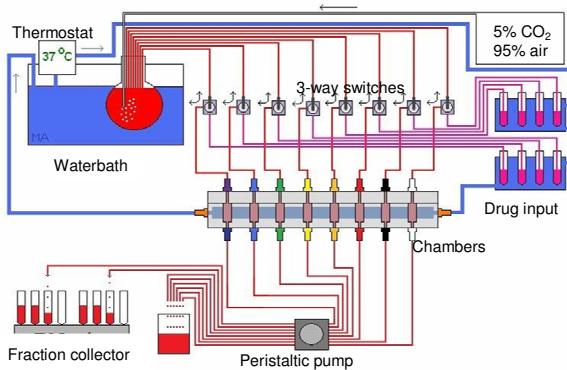
**IN VIVO EXPERIMENTS.** Fertilized eggs of Leghorn chickens were obtained (Bóly Hatchery Ltd.), then they were incubated until hatching under LD (14h light/10h dark) or DD environment at a constant temperature (37,8°C), humidity (55-70%), and automatical egg rocking (every 2 h, *Hemel Brutmaschine A70*). Hatchlings were kept under LD conditions for 6 weeks with *ad lib.* access to maize and water. Light was provided by white fluorescent lamps which produced light intensities of 400 Lux in the egg incubator and 600 Lux for hatchlings. In each experimental group, 3-3 chickens of the same developmental stage were decapitated in every 2 or 4 h. Samples were collected throughout 12, 24 or 36 h.

**IN VITRO EXPERIMENTS.** Chickens were decapitated on the first day of the experiment at 12:00. Pineal glands of 6 weeks old chickens (n=6) were chopped into 3-4 parts, then the

mixture of all tissue fragments were divided to 6 groups which were placed into 6 chambers of the multi-channel perfusion system (**Figure 3**). Pineal glands of chicken embryos (n=18) were placed in the 6 perfused chambers as whole (n=3 per chamber). The flow rate of tissue culture medium (*Sigma* Medium 199) was 4 ml/h at 37,8°C under LD (14h 225 Lux, 10h darkness) or DD conditions. Sampling started 30 h after the beginning of the experiment (*i.e.* second day at 18:00). Every 4 hours, tissue samples of one chamber were collected: the mixture of gland particles of post-hatch chicken were homogenized as one sample per chamber, while embryonic glands were collected as n=3 samples per chamber. In the PACAP-experiments, 10 nM PACAP-38 (*Sigma* A1439) was used on chambers #2-#6 for 60 minutes starting 2 h before the second sampling timepoint (20:00-21:00).

**Figure 3. Perfusion system (draft).**

The medium flows through a Teflon pipe with 1 mm inner diameter from the warmed container via the 3 way switch to the chamber (Ann Arbor Plastics), then through the peristaltic pump to the effluent container. Melatonin RIA of effluent medium was not always performed and those data are not discussed in this work. The drawing was made by Attila Matkovits.



**STATISTICAL ANALYSIS.** For standard curves, differences in SYBR fluorescence of (RT)-PCR products of serial diluted templates were determined with *Student's t-test* (one sample, homoscedastic, one tailed). In the *in vivo* experiments, differences between samples collected in different time points were assessed with one-way ANOVA followed by *Tukey's post hoc* test. The effects of different light/dark cycles on the 24 h pattern of clock gene expression was analysed with two-way ANOVA followed by *Student's t-test* (two sample, unequal variances, two tailed). In the *in vitro* experiments on pineal glands of 6 weeks old chickens, differences between samples were assessed with *non-parametric* tests. Differences between samples collected in different time points were assessed with *Wilcoxon's rank sum test*, and the effects of different light/dark cycles on the 24 h pattern of clock gene expression was analysed with *Mann-Whitney's u-test*. Differences between samples collected from chicken embryos *in vitro* in different time points were assessed with one-way ANOVA followed by *Tukey's post hoc* test. The effects of PACAP-38 treatment on the 24 h pattern of clock gene expression was analysed with two-way ANOVA followed by *Student's t-test* (two sample, unequal variances, two tailed).

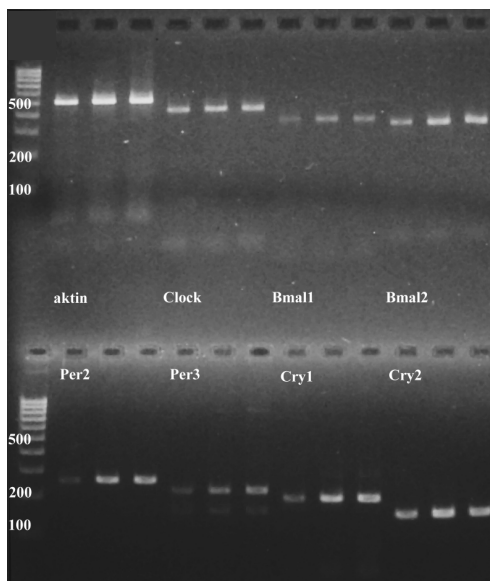
In all our statistics,  $p < 0.05$  values were considered to be significant.

# IDENTIFICATION OF CLOCK GENES IN THE CHICKEN PINEAL GLAND

## RESULTS

With the PCR primers designed by us we were succesful in all cases in obtaining discrete sized RT-PCR products from chicken pineal RNA samples (**Figure 4**). The length of the products was similar to the predicted values. mRNA-s of *bmal1*, *bmal2*, *per2*, *cry1* and *cry2* genes were detectable under all RT-PCR parameters tested, but *clock*, *per1* and *per3* products were seen only under certain PCR conditions. Secondary DNA bands were detected with *per1*, *per3* and *cry1* primers, where that of *cry1* was similar in size with the predicted genomic fragment. By sequencing both *cry1* products obtained from cDNA or genomic DNA, we have found 86% identity between the chicken and the mouse *cry1* gene fragment, which was in agreement with our expectations. The intronic sequence showed

98% identity with that of the chicken *cry1* gene which was published later and showed no homology with the mouse *cry1* gene. Next to *cry1*, our primers for *cry2* and *clock* worked also with a high reproducibility in the one-step semi-quantitative RT-PCR. The sequenmce of *cry2* and *clock* PCR productsshowed 99% identity with the templates used in primer design. In contrast to all other genes tested, the expression of *per1* could be detected only with very low reproducibility in our system, and there is still no data about chicken *per1* in the literature.



**Figure 4.** RT-PCR products of chicken pineal RNA samples amplified with clock gene specific primers, on agarose gel stained with ethidium-bromide.

## DISCUSSION

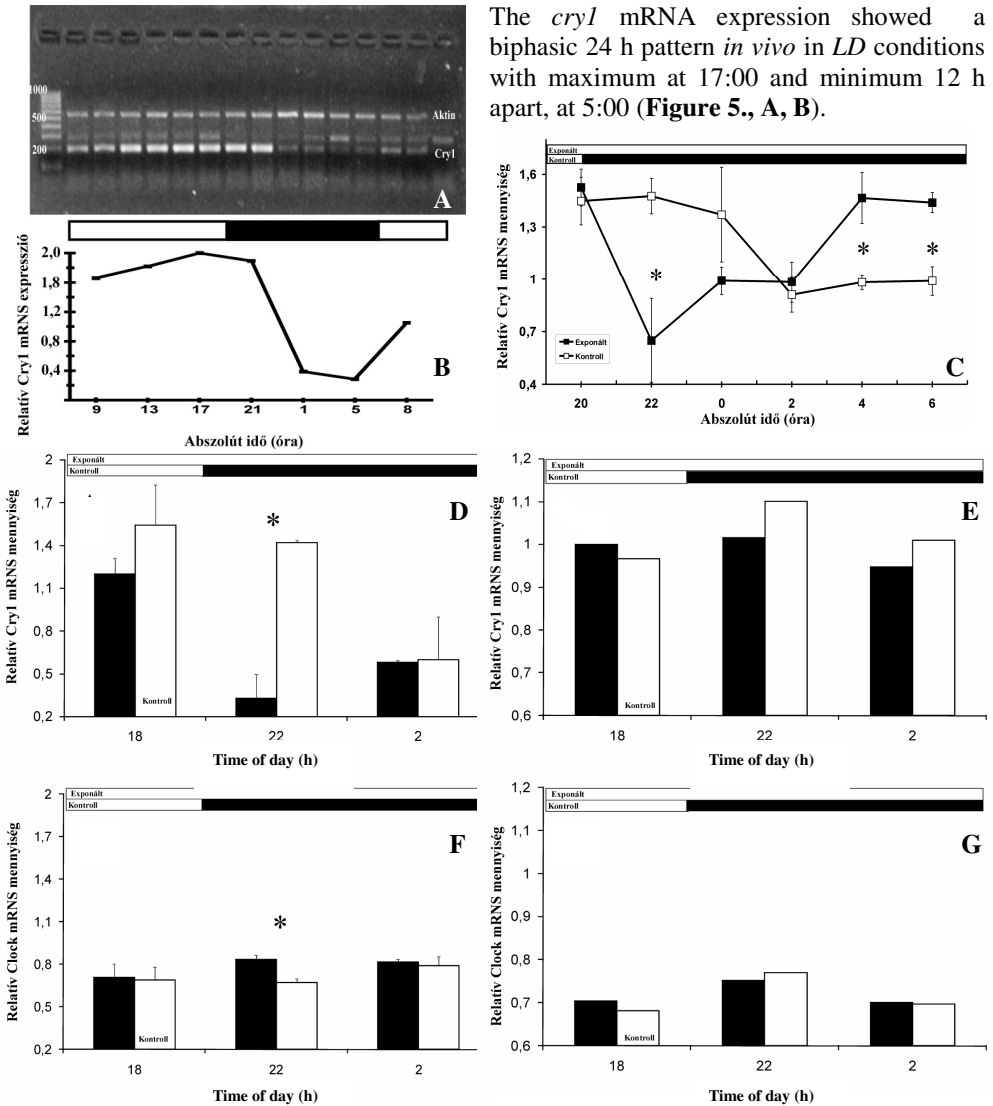
Our results are in agreement with all reports published meanwhile, and show evidence for the expression of chicken homologues of the clock genes in the pineal gland. Furthermore, the binding of chicken CLOCK/BMAL complexes to E-boxes, their transcriptional activator role, and its inhibition with co-expressed chicken CRY1 were also proven *in vitro*. All of these data suggest that the autonomic oscillations in chicken pineal oscillators are driven probably by a similar fedd-back mechanism of clock gene expression which was already described in the mammalian SCN.



# THE EFFECTS OF LIGHT ON THE CLOCK GENE MRNA EXPRESSION IN THE PINEAL GLAND OF 6 WEEKS OLD CHICKEN

## RESULTS

The *cry1* mRNA expression showed a biphasic 24 h pattern *in vivo* in LD conditions with maximum at 17:00 and minimum 12 h apart, at 5:00 (Figure 5., A, B).



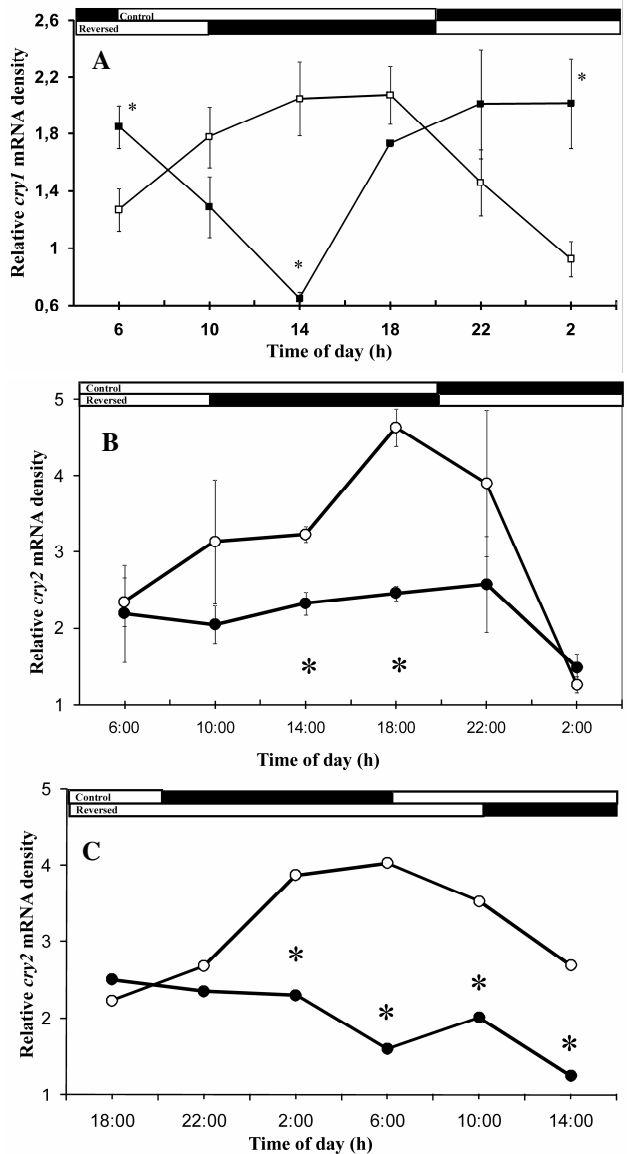
**Figure 5.** The effects of light on *cry1* (A-E) or clock (F-G) mRNA contents in the chicken pineal gland *in vivo* (A-D, F) and *in vitro* (E, G). Horizontal bars show environmental light/dark conditions (open: light, closed: dark). Data points and columns show the mean (+/- SEM, C, D, F) of relative mRNA expression in the *exposed* (closed) and *control* (empty) groups (C-G). Significant differences from control values are shown with \*.

Overnight light exposure *in vivo* resulted in a decrease of *cry1* mRNA levels at 22:00 (50%) and increase at 4:00 and 6:00 (140%), **Figure 5. C-D**; while *clock* expression at 22:00 was increased (115 % , **Figure 5. F**) if compared to control.

In contrast, *in vitro* overnight light exposure did not alter either *cry1* or *clock* expression (**Figure 5. E, G**).

Reversed light/dark cycle (10h dark/14h light, DL) *in vivo* resulted in an increase of *cry1* mRNA expression at 6:00 (140%), a decrease at 14:00 (30%), and again an increase at 2:00 (200%) if compared to control (**Figure 6. A**). *Cry2* expression in the DL group did not change between 6:00-22:00, but decreased by 18:00-2:00 to 70% (**Figure 6. B**). If compared to control, decreased *cry2* mRNA levels were measured between 14:00-18:00 (70% and 50%, **Figure 6. B**).

Reversed light/dark cycle *in vitro* resulted in a steady decrease of *cry2* mRNA contents throughout the experiment (to 50%, **Figure 6. C**). In the control group, the 24 h pattern of *cry2* expression showed an increase by 6:00 (in darkness) followed by a decrease. If compared to control, *cry2* mRNA levels in the DL group were decreased between 2:00-14:00 (40%-70%).



**Figure 6.** The effects of reversed light/dark cycle on the 24 h alterations of *cry1* (A) and *cry2* (B-C) mRNA contents of chicken pineal glands *in vivo* (A-B) and *in vitro* (C). Figure labelling is identical to that of figure 5.

## DISCUSSION

Under *LD* conditions, the expression pattern of clock genes in the chicken pineal is similar not to the mammalian pineal but to the mammalian SCN. Maximal *cry1* mRNA contents are seen *in vitro* around the midpoint of the light phase (data from the literature) or *in vivo* around the light-to-dark transition (**Figure 5. A-C**), indicating that *cry1* expression is induced by light via both retinal and pineal photoreceptors. The delay of the *in vivo* pattern compared to that of seen *in vitro* suggests a modulatory role for the neurohumoral environment. This delay is 180° in the case of *cry2*: the *in vivo* pattern of *cry2* mRNA expression, which is similar to *cry1* (**Figure 6. A-B**, control), is driven by neurohumoral stimuli, while *in vitro* the 180° delayed *cry2* phase curve (**Figure 6. C**, control) reflects the effects of signalling pathways related exclusively to pineal oscillators and photoreceptors. The 24 h pattern of *clock* mRNA expression is also similar to that of *cry1* *in vivo* in LD, however, with much smaller amplitude (data from the literature).

*Light exposure at night* causes a transient drop of *cry1* mRNA levels in 2 hours *in vivo* (**Figure 5. C-D**). Similar to that seen in the mammalian SCN clock, the light dependent induction of *cry1* gene in the chicken pineal model can be exclusively indirect. mRNA levels of *clock* were also altered, however, in an opposite way (**Figure 5. F**), showing that unanticipated light stimulus (*i.e.* at night) evokes various transcriptional regulatory events also in the chicken pineal gland which leads to different responses in the expression of the clock genes. In contrast, a similar light exposure did not alter the expression of *cry1* *in vitro* within 6 h (**Figure 5. E, G**), suggesting that neurohumoral stimuli are necessary for a rapid adaptation to the changes in environmental LD conditions. On the other hand, *cry2* did show alteration during *in vitro* light exposure at night (**Figure 6. C**, 18:00-6:00), further supporting the idea that in spite of the similar *in vivo* pattern of *cry1* and *cry2* mRNA expression, they are controlled by pineal photoreceptors with different mechanisms.

*Under reversed light/dark cycle (DL)* the pattern of *cry1* expression was inverted within 24 h *in vivo*, with still elevated mRNA levels during the illuminated phase (**Figure 6. A**). The indirect way of light dependent induction of *cry1* is supported not only by the transient drop during the first cycle of light phase at night (22:00, **Figure 5. C-D**), but also by an increase in the last hours of the first dark phase during the day (18:00, **Figure 6. A**). These data suggest that *in vivo* an unanticipated exposure to light influences those signalling pathways, which are able not only to alter *cry1* expression, but also to reset the phase curve of the molecular oscillator in the chicken pineal gland within 24 h to the new LD conditions.

The *cry2* expression showed opposite light dependent changes *in vitro* (**Figure 6. C**) if compared to *in vivo* data (**Figure 6. B**): in the same time interval (22:00-6:00) lower mRNA levels were measured during light (DL), than during darkness (LD, **Figure 6. C**). This suggests again that pineal photoreceptors and neurohumoral stimuli use different pathways to regulate *cry2* expression.

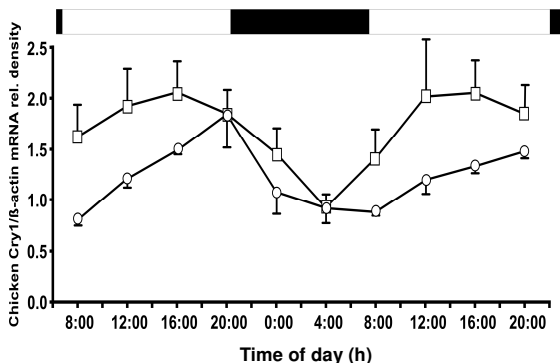
Unlike *cry1*, the rhythm of *cry2* expression was not inverted during the first 24 h of reversed LD conditions (**Figure 6. B**). Lower *cry2* mRNA contents were measured at the

same timepoint in both groups (DL vs. LD, 2:00, **Figure 6. B**), regardless to the lighting schedule (light vs. dark). This shows that unanticipated light may evoke various signalling mechanisms with opposite effects: neuroendocrine stimuli may induce (*in vivo*, **Figure 6. B**) and pineal photoreceptors may repress *cry2* expression (*in vitro*, **Figure 6. C**). The interaction of both mechanisms may corrupt the regulatory role of the 180° phase shifted molecular oscillator on *cry2* transcription during the first reversed LD cycles, which means not a *cry1*-like rapid adaptation for the *cry2* mRNA rhythm (**Figure 6. A-B**). Our speculation about the causes of this adaptational delay of the *cry2* rhythm *in vivo* was supported by our *in vitro* DL data: the *cry2* expression showed a steady decrease throughout the whole first cycle of reversed LD conditions if compared to control (**Figure 6. C**), which indicates a role for pineal photoreceptors in *cry2* repression. Based on our results, we hypothesise that unanticipated light exposure affects the chicken pineal clock *in vivo* through (1) pineal photoreceptors which can be monitored at the level of *cry2*, and through (2) the retina via neuroendocrine stimuli which can be examined at the level of *cry1* and *clock* mRNA expression.

## THE DEVELOPMENT OF THE RHYTHMIC PATTERN OF CLOCK GENE EXPRESSION IN THE EMBRYONIC CHICKEN PINEAL GLAND

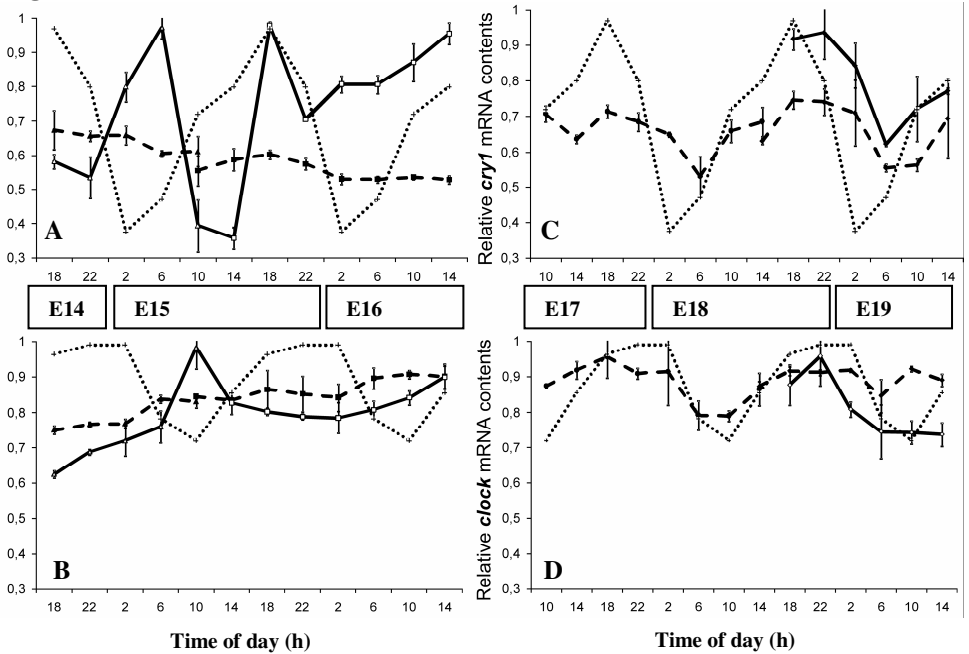
### RESULTS

The *cry1* mRNA expression showed a biphasic 24 h pattern *in vivo* in the pineal gland of chicken embryos incubated in LD conditions: with maximum at 20:00 and minimum 12 h apart, at 8:00 (**Figure 7**). We found similar results in 6 weeks old chickens: maximum was seen at 16:00 and minimum was measured at 4:00.



**Figure 7.** Chicken pineal *cry1* mRNA expression *in vivo* under LD. Horizontal bars show environmental light/dark conditions (open: light, closed: dark). Data points show the mean (+ or - SEM) of relative mRNA expression in groups of embryos (circles) and 6 weeks old chickens (squares).

In chicken embryos incubated under *DD* conditions *in vivo*, *cry1* expression showed between embryonic days 13-16 a steady decrease, while *clock* mRNA was detected from day 14 with a steady increase (**Figure 8. A, B**). *Cry1* mRNA started to increase by day 17. By the beginning of day 18, both *cry1* and *clock* expression decreased (by 6:00), which was followed by an increase (**Figure 8. C, D**). By the beginning of day 19 there was again a decrease seen in *cry1* mRNA contents (at 6:00), again followed by an increase (**Figure 8. B**). Data on *clock* expression did not show significant changes on day 19 (**Figure 8. D**).



**Figure 8.** *Cry1* (A, C) and *clock* (B, D) mRNA contents of pineal glands of chicken embryos incubated under *DD* conditions. Dashed and continuous lines show *in vivo* and *in vitro* data, respectively. Data points show the mean (+/- SEM) of relative mRNA expression. The dotted line shows *in vivo* data obtained from 6 weeks old chickens kept in LD conditions. Quadrangles show the embryonal stage (e.g. E15 = day 15 of embryonal development).

In chicken embryos incubated under *DD* conditions *in vitro*, *cry1* expression showed on day 15 a peak at 6:00 and 18:00 (**Figure 8. A**), while the expression of *clock* peaked at 10:00 (**Figure 8. B**). On day 16 both *cry1* and *clock* mRNA levels showed a steady increase (**Figure 8. A, B**). On day 18 both *cry1* and *clock* expression peaked at 22:00, followed by a drop on the following day at 6:00 (**Figure 8. C, D**).

## DISCUSSION

Recent data have shown that the pineal expression of the directly light-inducible *per2* is rhythmic in E18 stage chicken embryos incubated under LD *in vivo*. According to our results, the expression of the not directly light-inducible *cry1* is also rhythmic under similar experimental conditions (**Figure 7**). The *cry1* phase curve was very similar to that seen in 6 weeks old chicken regarding both the time of peak values and the amplitude. These data suggest that by the stage of E17 there has already developed a functional molecular oscillator which works synchronized to environmental zeitgebers.

The role of the neurohumoral environment in initiating circadian oscillations of the pineal clock was investigated in chicken embryos incubated under DD conditions. Between E13-16 stages *in vivo*, pineal *cry1* expression was continuously decreasing while *clock* mRNA levels increasing (**Figure 8. A, B**, dashed lines). No daily rhythm was seen at the tissue level which may refer either to a lack of synchrony among oscillatory units or to the complete absence of any oscillation at this stage. Furthermore, the opposite changes in *cry1* and *clock* mRNA amounts suggest different transcriptional regulation.

Between E16-19 stages, the expression pattern of both genes has changed at the tissue level: in contrast to the almost steady levels before E13, episodic increase and decrease of mRNA contents were seen within a 24 hour period (**Figure 8. C, D**, dashed lines). Furthermore, *clock* mRNA expression showed from day 17<sup>th</sup> not an opposite, but rather a parallel phase curve if compared to that of *cry1* which resembles the typical phase-relationship between the two genes' expression patterns seen in 6 weeks old chickens. These data suggest that changes in the transcriptional regulation of clock genes develop around the E16 stage which either starts or synchronises the oscillators to each other. This developmental event is under neuroendocrine or paracrine control and needs no environmental stimuli.

For both genes the mRNA rhythm seen between E17-19 under DD showed lower amplitudes if compared either to those of 6 weeks old chickens or to those of E17 embryos incubated under LD (**Figure 7**). This suggests that under LD there is a higher proportion of pinealocytes and photoreceptors which become synchronised to each other. Based on our data we speculate that signalling pathways activated by periodic environmental stimuli are needed not to start the oscillation rather to enhance a more extensive intercellular synchronisation which is needed to produce a high amplitude rhythm at the tissue level. The same role of LD environment was demonstrated during postnatal development of the mammalian SCN neurons. Interestingly, oscillatory units in the zebrafish pineal clock model are unable to work synchronized unless there is at least one single light pulse during ontogenesis. In the light of these data it becomes more obvious that the avian pineal gland serves as a special clock model for the study of neuroendocrine and paracrine factors responsible for the development of circadian rhythms. Unlike in fish, synchronised oscillation may start in the absence of light stimuli, and unlike in mammals, it starts in the absence of maternal zeitgebers.

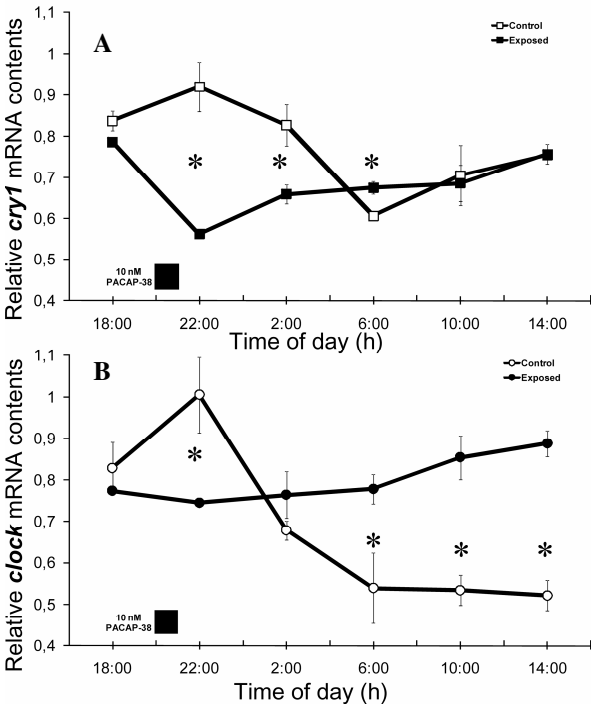
In contrast to our *in vivo* results, we found *in vitro* in all developmental stages significant episodic alterations of clock gene expression in the pineal glands taken from chicken

embryos incubated under DD (**Figure 8**, continuous line). Differences between *in vivo* and *in vitro* data may reflect the different effects of local (paracrine) and neuroendocrine stimuli. The activatory effects of episodically occurring intra- or intercellular signals may be masked by neuroendocrine stimuli. Unlike *cry1*, phase curves of *clock* expression are similar *in vivo* and *in vitro*, suggesting that *clock* transcription is regulated mainly by the changes in intra- or intercellular processes.

Our *in vitro* experiments have also proven that during the ontogenesis of the chick pineal gland, key changes occur between E14-17 stages in the transcriptional regulation of clock genes, even in the absence of periodic environmental stimuli. Unlike the phase curves on day 13 or 14, in E17 pineals both genes showed similar 24 h mRNA patterns with peak values at 22:00 (**Figure 8. C, D**). Furthermore, their phase-relationships were similar to what was seen *in vitro* under DD conditions in the pineal glands of 6 weeks old chicken previously kept in LD (data from the literature). These data support that circadian molecular oscillators may start working synchronised already from stage E17 also *in vitro* in the absence of any periodic environmental or neuroendocrine stimulus. This suggests that the *in vivo* rhythm of clock gene expression developing at the same stage (E17) is driven not by the neuroendocrine environment. However, based on our data it can not be excluded if an episodic, light-independent neurohumoral stimulus would be needed to initiate or evoke molecular oscillations.

### THE EFFECTS OF PACAP-38 ON *CRY1* AND *CLOCK* EXPRESSION IN THE CHICKEN PINEAL GLAND

**Figure 9.** Effects of 10 nM PACAP-38 treatment *in vitro* on the 24 h patterns of *cry1* (A) and *clock* (B) mRNA expression in pineal glands of chicken embryos incubated in DD. Data points show the mean (+/- SEM) of relative mRNA expression in the control (empty) and exposed (closed) groups. The black box shows the time period of 60 min for PACAP exposure. Significant differences from control values are shown with \*.



## RESULTS

Pineal glands of E17 chicken embryos previously incubated in DD were placed to *in vitro* DD environment, where tissue samples were treated with 10nM PACAP-38 for 60 min on day 18 at 20:00.

By 22:00, both *cry1* and *clock* expression was decreased if compared to control (**Figure 9**). After 6:00, *cry1* mRNA contents were not much different from control values, while *clock* mRNA levels exceeded that of the controls.

## DISCUSSION

The PACAP treatment, which was similar in dose and timing to what was effectively used in the mammalian SCN model, resulted in a decrease of both *cry1* and *clock* mRNA contents in the pineals within 2 h after the onset of exposure (**Figure 9**). Although we did not test the effects of other doses or schedules of PACAP treatment, the fact that both genes showed similar response in their mRNA contents suggest that PACAP may indeed have some role in synchronising molecular oscillators also in the chicken pineal clock model. Previous data on the presence of PAC<sub>1</sub> and VPAC<sub>1</sub> receptors, signalling pathways similar to the mammalian SCN, and PACAP-immunopositive nerve fibres originating from the *trigeminal ganglion* and terminating on the pineal gland further support our hypothesis.

## **NOVEL FINDINGS**

- (1) Effects of *in vivo* unanticipated light exposure mediated by pineal photoreceptors can be monitored at the level of *cry2*, and those mediated by the retina via neuroendocrine stimuli can be examined at the level of *cry1* and *clock* mRNA expression in the chicken pineal clock model.
- (2) The transcriptional regulation of *cry1* and *clock* genes can adapt rapidly (within 2 h) to unanticipated light-dependent neuroendocrine stimuli. Unlike *period* genes, the light-dependent induction of *cry1* and *cry2* clock genes is indirect also in the chicken pineal clock model.
- (3) From day 16 of embryonic development, neuroendocrine and/or paracrine stimuli are responsible for the changes in the transcriptional regulation of clock genes securing synchronised oscillations in the developing chicken pineal clock. The periodic alterations of the physical environment (light, temperature) are not necessary in this process.
- (4) PACAP may have a role in synchronising molecular oscillators also in the chicken pineal clock model.



## PUBLICATIONS

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- (3) *András Dávid Nagy* and Valér Csernus: *Cry1* expression in the chicken pineal gland: effects of changes in the light/dark conditions. - *Gen. Comp. Endocrinol.* 152: 148-153. 2007. [IF: 2.562]
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### BOOK CHAPTERS

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ABSTRACTS PUBLISHED ON INTERNATIONAL CONGRESSES: 13

ABSTRACTS PUBLISHED ON NATIONAL CONGRESSES: 14

SEMINARS, WORKSHOPS, CONGRESS ORGANIZING ACTIVITY: 5

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