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

Siri Kommedal, M.D.

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Supervisor: Csernus Valer MD, PhD,

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List of abbreviations:

- LD light/dark
- DD dark/dark
- DL dark/light (reversed cycle)
- ZT zeitgeber
- ZT x zeitgeber time x
- CCG clock controlled genes
- IARC International Agency for Research on Cancer
- ipRCG Intrinsically photosensitive retinal ganglion cells
- RHT Retino-hypothalamic tract
- SCN supra-chiasmatic nucleus
- PACAP pituitary adenylate cyclase activating polypeptide
- NE norepinephrine
- AANAT arylalkylamine-N-acetyltransferase
- MT1 mammalian melatonin receptor 1
- MT2 mammalian melatonin receptor 2
- PT pars tuberalis
- Mel1a chicken melatonin receptor 1a
- Mel1b chicken melatonin receptor 1b
- Mel1c chicken melatonin receptor 1c
- HIOMT hydroxyindol-O-mehtyltransferase
- LD +4 four hour phase delay
- ED x embryonic day x

1) INTRODUCTION

1.1) Biological Rhythms:

Most biological processes are periodically recurring, and rhythmic in nature. Biological rhythms are phenomena that recur in living organisms with a typical period length. These help us to anticipate periodical changes in the environment such as light, temperature, food availability etc. (Zeman M., 2011). There exists a great variety of biological rhythms such as autumnal effects on plants, growth pattern of fungi, the menstrual cycle, sleep-wake cycles and hormonal releasing cycles.

Biological rhythms can be classified according to their duration/length. Cycles that last longer than a day are called infradian rhythms such as the menstrual cycle. Cycles that last for less than a day are called ultradian rhythms and these include rhythm such as breathing and heart rate regulation. Cycles that last for roughly a day are called circadian rhythms (circa-approximately, diem-day), such as the sleep/wake cycle or the regulation of many hormonal levels e.g. cortisol in the blood. These physiological rhythms show a variance in their expression: some have a diurnal, and others have a nocturnal zenith. Examples of rhythms with a diurnal zenith are the release of: serotonin, catecholamine, cortisol, renin-angiotensin, aldosterone, and ADH. Examples of those with nocturnal zenith are the release of: melatonin, acetylcholine, dopamine, nitric oxide, endorphins, and antioxidants.

Circadian rhythms are amongst others regulated by the light/dark (LD) cycles that result from the Earth's rotations (Zeman M., 2011) (Stehle JH, 2003). These rhythms are found everywhere in biology and all have some basic elements in common:

1) Similarly to other biological rhythmic activities, they are controlled by an endogenous mechanism. This means that even when the organism is placed within a non-periodic environment such as constant darkness (DD) with constant temperature and humidity the rhythm will keep running with a period which is close to 24 hours (Book, Refinetti, 1999) (Review, Bell-Pedersen D, 2005). Based on the nature of the internal

mechanism, the period time of a given biological rhythm can be modified only within very narrow limits.

2) The phase of the rhythm, however, can be easily shifted by a rhythmic environmental stimulus, called a Zeitgeber (ZT) or time giver (Book, Refinetti, 1999). This means that the rhythms have to quickly and appropriately respond to environmental stimuli in such a manner that the rhythm shifts to occur at the desired time. This is driven by biological oscillator mechanisms.

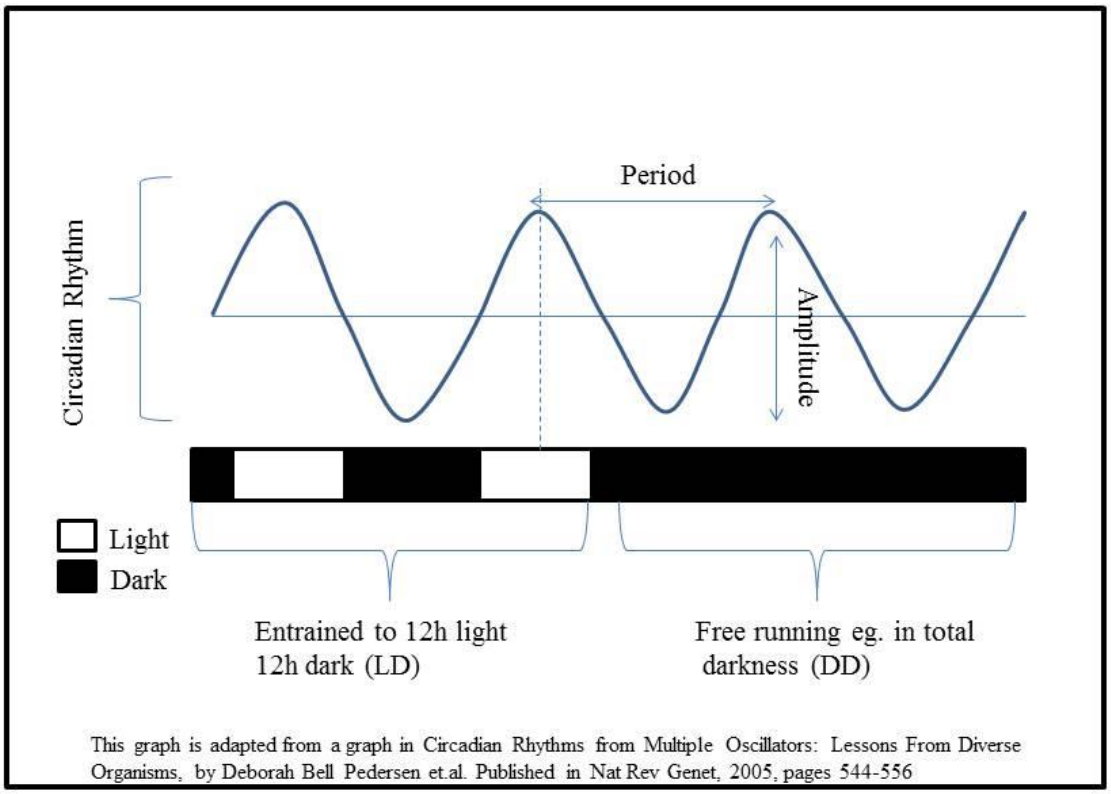

Fig. 1) Graphic representation of a circadian rhythm and some of its key features. The period of the rhythm is roughly 24 hours in case of a circadian rhtyhm, even under free running conditions. The amplitude depends on the rhythm studied.

All biological rhythms, including circadian ones, have some common basic features (fig. 1). The phase of the rhythm is the relative difference of the peak compared to an external event. In other words it refers to the difference in time between e.g. the light part of the day and the peak of gene expression. Many studies look at how the phase of the rhythm changes with changes in the environment. Another important feature of circadian rhythms is its period time (V. Csernus, 2003). The period time of all circadian rhythms is roughly 24 hours long, and this can be modified only within a narrow range. In case of the circadian rhythm, this is in the magnitude of 20-30 minute deviation. In addition to this a biological clock can and will run

even without the presence of a Zeitgeber. Without a Zeitgeber the clock will not be synchronized, but it will run free, based on its internal period time. This means, as mentioned above, that even under free running or constant conditions such as DD the period will be close to 24 hours long.

1.2) The Circadian Clock:

Circadian rhythms are driven by so called biological clocks, a term which was coined in the 1960s. Circadian clocks contain all the components of the pace-maker oscillators usually within one cell, making them capable of controlling the circadian time with amazing accuracy (Review, Bell-Pedersen D, 2005). The oscillating system relies on a combination of positive and negative feed-back-loops. These feedback loops require only two components (fig. 2):

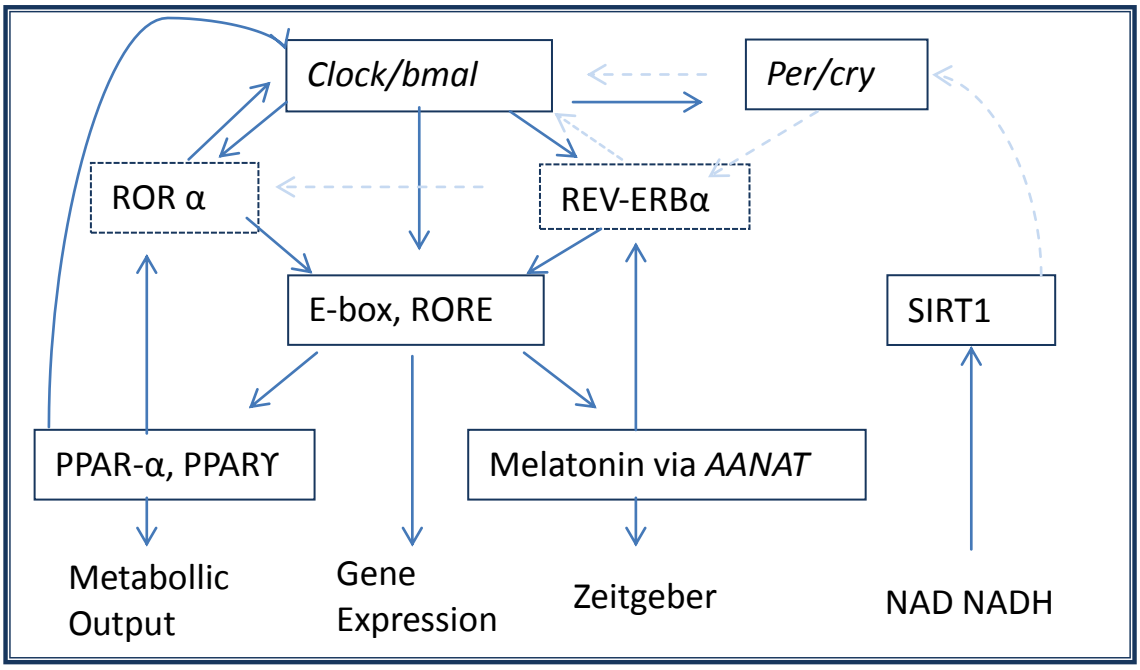

Fig 2. Schematic outline of the molecular oscillator. *Clock/bmal1* activates the *cry* and *per* promoters. Next there is transcription/translation of *per* and *cry*, before they enter the nucleus and in turn inhibit the *clock/bmal1* activity. The key output is an E-box mediated transcriptional activation of hundreds of target genes on a daily basis such as *AANAT*.

1) The first complex, the activation complex, is composed of genes which activate the expression of other genes; these are called the positive regulators. In the circadian clock of mammals this is made up most frequently of the clock genes *clock* and *bmal* as a heterodimer (Gekakis N, 1998). *Clock/Bmal* will enter the nucleus and bind to an E-box promoter and thereby induce transcription. *Clock/Bmal* can induce the transcription of the negative complex, but also of clock controlled genes (CCG) by acting directly on E-box sequences in the promoters of output genes which in turn affect the rhythmic expression of CCG (Review, Bell-Pedersen D, 2005).

2) The second, inhibitory complex, is composed of genes that act as negative regulators. They will counteract the function of the activation complex. This complex is composed of a *per* and *cry* heterodimer (Miyazaki K, 2001). *Per/cry* will interact with *clock/bmal* and inhibit their activity. The inhibition is terminated by phosphorylation induced degradation of clock proteins which, in turn, allows for the initiation of a new cycle (Review, Bell-Pedersen D, 2005). This requires a secondary feedback loop which is regulated by REV-ERBα and RORα proteins. The necessary time delay of the feedback loops, which is responsible for making the period 24 hours long, involves a complex set of post-translational mechanisms (Akashi M, 2002;) (Scheper T, 1999).

1.3) The physiological importance of the circadian clock:

Circadian clocks play a key role in maintaining normal homeostasis, and participate in various physiological and pathophysiological mechanisms (Eckel-Mahan K, 2013). Circadian rhythms are found in a variety of physiological processes with daily fluctuations with an average of 20-30% change in amplitude. Up to 90% of cell-specific genes may show a 24 hour pattern in their expression showing how wide-spread rhythms are within the cell. In addition to this, fundamental cellular processes, such as the cell cycle, have been shown to be under the control of molecular mechanisms suggesting a general "housekeeping" role for the circadian clock in biology.

There are also clear correlations between the expression of circadian rhythms, clinical symptoms and disease. Cardiovascular emergency cases and sudden death most frequently occur in the early morning hours when there is increased stress on the body with an increase in the blood pressure and pulse (Wang H, 1995). Diabetic patients more frequently have problems with controlling the glucose levels in the early morning as this is the time when insulin like hormone levels are the highest (Bizot-Espiard JG, 1998).

When it is made clear that regulation of circadian rhythms, or disruption of these rhythms, can lead to human diseases it is easier to understand why this is an important field to study. The novel branch of medical sciences that takes the circadian rhythms into consideration during therapy is named chronotherapy. Chronotherapy is based on the idea that clinical therapy can be made more efficient, and will require a lower dosage of drugs and thus fewer side effects if the treatment is applied at the time of the appropriate daily phase of neuro-humoral condition (Lemmer B, 1987). This idea has been utilized in the treatments of some cancers, and it is becoming a widely used approach to treat a variety of other disorders.

1.4) Jet lag or phase shift of the circadian rhythms:

A fundamental feature of all circadian rhythms is that they follow the changes in the environmental periodic stimuli by a shift in the phase of the rhythm. This is seen in people who take transatlantic flights. The phase advance or delay in the light schedule triggers a change in the circadian rhythmic expression of clock genes and, consequentially, other genes such as genes involved in melatonin synthesis (Erren T.C., 2009). Chronodisruption, i.e. incongruence between the endogenous internal clock time and the rhythmic environmental stimuli, can lead to acute symptoms such as fatigue, somnolence, gastrointestinal problems etc. (Eckel-Mahan K, 2013). People that are repeatedly exposed to phase shifts, for example shift workers, have been demonstrated to have an increased risk of metabolic syndrome, cardiovascular diseases and certain cancers (Boivin D.B., 2007) (Karlsson B., 2001) (Scheer F.A., 2009) (Rajaratnam SMW, 2001) (Davis S., 2006). Studies done on shift workers have demonstrated that they show an increased risk in obesity, and gain weight more easily than non-shift workers (Eckel-Mahan K, 2013). They also have a clearly increased risk of breast cancer, and perhaps colon cancer and prostate cancer (Davis S., 2006). The correlation between disrupted circadian rhythmicity in shift workers and increased cancer rate is so strong that in 2007 the International Agency for Research on Cancer (IARC) classified shift-work as probably carcinogenic to humans (Erren T.C., 2009). This has triggered a lot of work on the phase advance and phase delay of the circadian rhythms and their adverse effects on health.

By plotting the effect light has on the circadian rhythm at different times of the day, scientists have come up with a phase response curve which show, what effect light has at different times of the night and day (Zee PC, 2013). The effect of light on the circadian clock, the expression of clock genes and output of the clock depends on the time at which the light is applied (fig. 3). Light which is applied at early night will cause a phase delay of the rhythm, whilst light which is applied during the day has little or no effect on the circadian clock (Hatori M, 2011) (Korf HW, 2003). An exposure to light at late night will cause a phase advance of the rhythm (Dijk Derk-Jan, 2012) (Korf HW, 2003). This can be seen both at the level of transcriptional activity of circadian clock genes, and at the level of melatonin release from the pineal gland (Cassone, 1990). Experiments on mice have shown that the circadian system responds faster to a phase delay of the rhythm than a phase advance (Pfeffer M, 2012). This may in part be due to the fact that a phase delay of the rhtyhm entails a prolongation of lights on, which

typically inhibits the locomotor activity of mice which has been studied (Pfeffer M, 2012). Thus the difference in the duration of the resetting of the rhythm may not be only due to different moelecular mecahnisms, but may in mice be directly attributed to the effect of light.

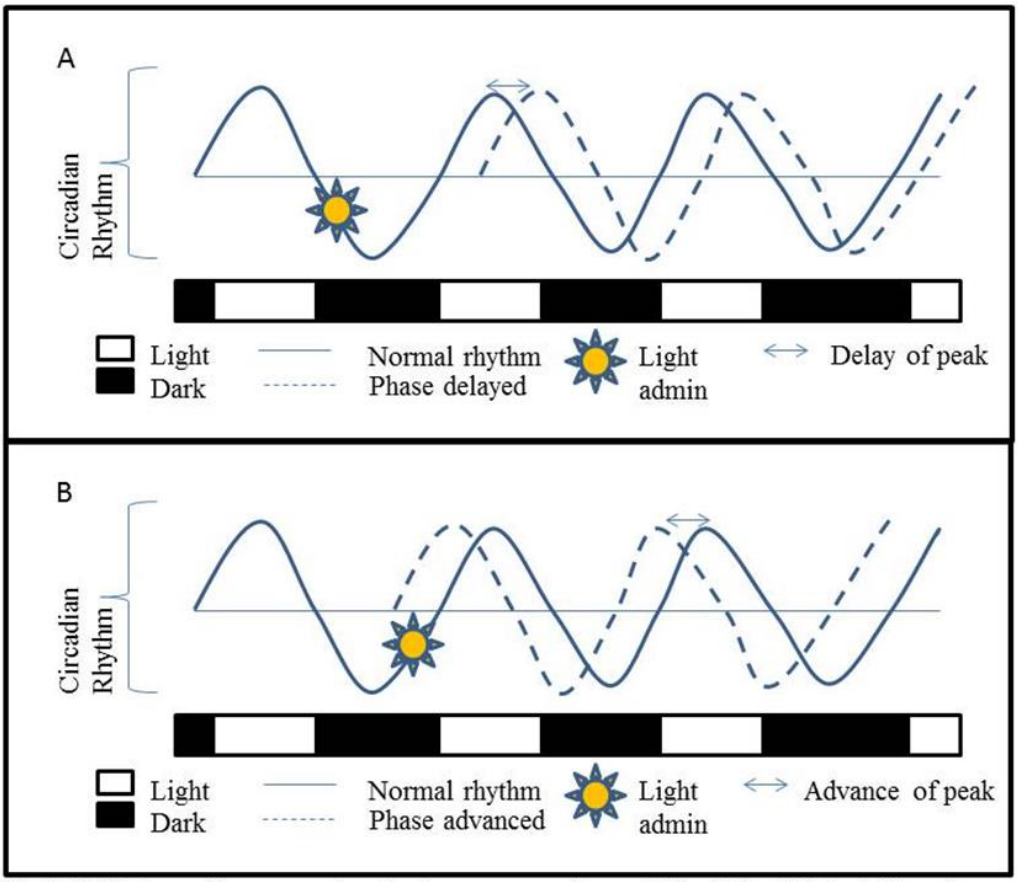

Fig.3. A) This is a graphic representation of a phase response to changes in light. Light which is administered at early night causes a phase delay of the rhythm. B) When light is administered at late night it causes a phase advance of the rhythm.

1.5) Models of the circadian clock:

The best way to study internal rhythms and to examine changes in these rhythms is if changes in the environment are set constant, e.g. under DD. As early as in in 1729 it was noted by De Marian that heliotrope plants moved their leaves in the same rhythmic pattern under DD as they would to follow the sun during normal (LD) conditions (A., S.., 1995). In other words it has been known for over 200 years that there exists an inner clock mechanism that drives a multitude of daily rhythms, and that this can be entrained to the environment.

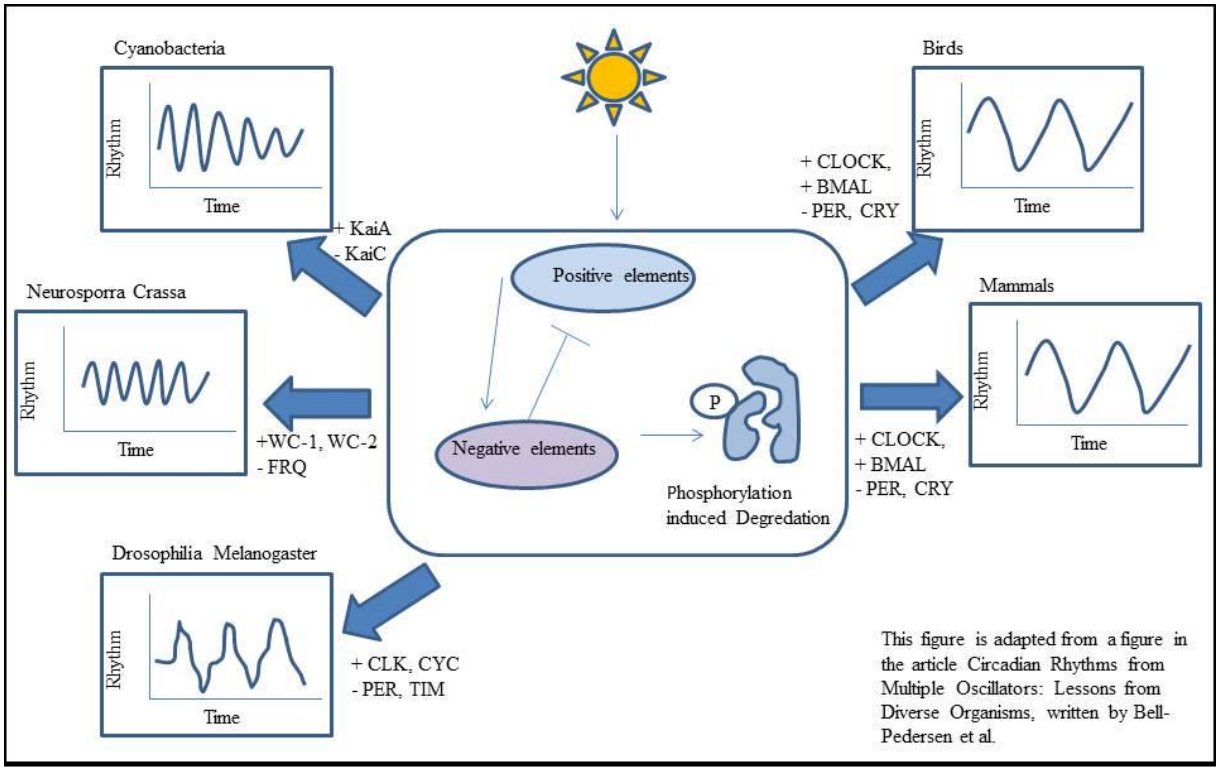

Fig 4) Although the underlying circadian mechanisms are the same across species the exact clock genes involved vary from species to species. The rhythms which we study may also be different across species. In mammals we frequently study locomotion as an output of the rhythm. In birds melatonin is sometimes studied. In Cyanobacteria the rhythm of bioilluminecencse may be studied. In Neurosporra Crassa the rhythm of growth. In Drosophilia Melanogaster locotmotion is also frequently studied. Across species we can directly study both the clock genes and the output of the clock.

Classical laboratory models to study the inner clock mechanisms and its rhythms include: the study of growth dynamics of fungi, the metabolism of unicellular eukaryotic organisms, the growth and flowering of plants, timing of the metamorphosis of insects, and the locomotor and endocrine functions of vertebrates (A., S., 1995) (Review, Bell-Pedersen D, 2005). The

underlying core mechanisms are similar across species, but the exact clock genes involved differ, see the figure above (fig. 4) for a short summary of some clock genes in different species. Common features among all of these are that there is a roughly 24 hour rhythm found also under DD, whose phase can be adjusted by environmental factors, and that the rhythm is regulated by positive and negative feedback loops. Differences include the exact period length of the rhythm, diurnal or nocturnal zenith, and the factors, which entrain the rhythm, as well as the localization and interaction of the components of the clock mechanism.

One of the most important environmental stimuli is the light/dark cycle (Rohling J H. T., 2011) (Chen Rongmin, 2008) (Stehle JH, 2003) (Korf HW, 2003). In mammals light passes through the retina and via the intrinsically photosensitive retinal ganglion cells (ipRGC) the information is passed to non-image forming brain areas (Dijk Derk-Jan, 2012) (Korf HW, 2003). The retinohypothalamic tract (RHT) ends on the suprachiasmatic nucleus (SCN), the ablation of which results in disruption of rhythmicity in key physiological functions (Korf HW, 2003). These fibers contain both pituitary adenylate cyclase activating polypeptide (PACAP) and glutamate, and it is believed these neurotransmitters signal time of day (Korf HW, 2003). In mammals, the SCN is the primary pacemaker for circadian rhythms, and is the only oscillators in mammals, which is known to be able to entrain to light dark conditions (Bell-Pedersen D, 2005). SCN neurons are unique in that their oscillators can stay synchronized with each other even without the presence of any Zeitgeber, due to their abundant intercellular connections. From the SCN the phase-signal will be transmitted to the nearby hypothalamic centers that regulate a multitude of physiological functions such as body temperature, sleep/wake cycles, locomotor activity etc. In mammals, the SCN is connected to the pineal gland through a multisynaptic neuronal chain which terminates in postganglionic fibers that release norepinephrine in a rhythmic manner, which in turn stimulates the rhythmic release of melatonin (Korf HW, 2003).

At the molecular level 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 signaling pathways (Bell-Pedersen D, 2005). 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") (Zatz, 2002)

2) it is needed in the regulation of photoperiod dependent biological processes (*e.g.* seasonal breeders).

1.6) The avian pineal gland as a circadian clock model:

In mammals, the master pacemaker is found within the SCN. The SCN is not an easy organ to study once it is isolated. In most birds, however, the pineal gland is the central pacemaker. This is a favorite experimental model to study circadian rhythms because it has several advantages to the mammalian system (fig 5):

1) It is directly light sensitive. Being placed close to the calvaria, a translucent thin bone, light can directly act on the pineal gland. (A.D. Nagy S. K., 2008)
2) The amplitude of melatonin release can be influenced *in vitro* both by direct light exposure and via adrenergic stimulation (α2) (Barrett RK, 1995) (Binkley SA, 1978) (Faluhelyi N, 2005) (Takahashi JS, 1980), (Csernus, 2006). In birds, a diurnal animal, the effect of norepinephrine (NE) on melatonin is opposite to that seen in mammals. In birds, NE is produced during the light phase and acts to inhibit the formation of *AANAT* (arylalkylamine-N-acetytransferase) and melatonin during the day (Review Zeman M, 2011) (Zatz M, 2000).
3) Unlike the pineal glands of other vertebrates, the avian pineal gland can oscillate *in vitro* under DD conditions for greater than five cycles making it easily comparable to the mammalian SCN (Review Zeman M., 2011) (Csernus, 2006).
4) As opposed to in mammals, pinealectomy in birds may completely abolish many circadian rhythms.
5) It provides an *in vitro* model which can be completely isolated from the surrounding neuroendocrine and paracrine environment whilst still maintaining its histomorphological structure, which is not possible with the mammalian SCN (Falcon J, 1989) (Natesan A, 2002) (Zimmerman NH, 1975).
6) Embryonic chicken are “isolated” in eggs and thus are not affected by maternal hormonal rhythms during ontogenesis. Additionally even embryonic pineal glands can be used in *in vitro* studies, making this a great model for studying environmental and genetic factors involved in the development and synchronization of the circadian system under controlled conditions.

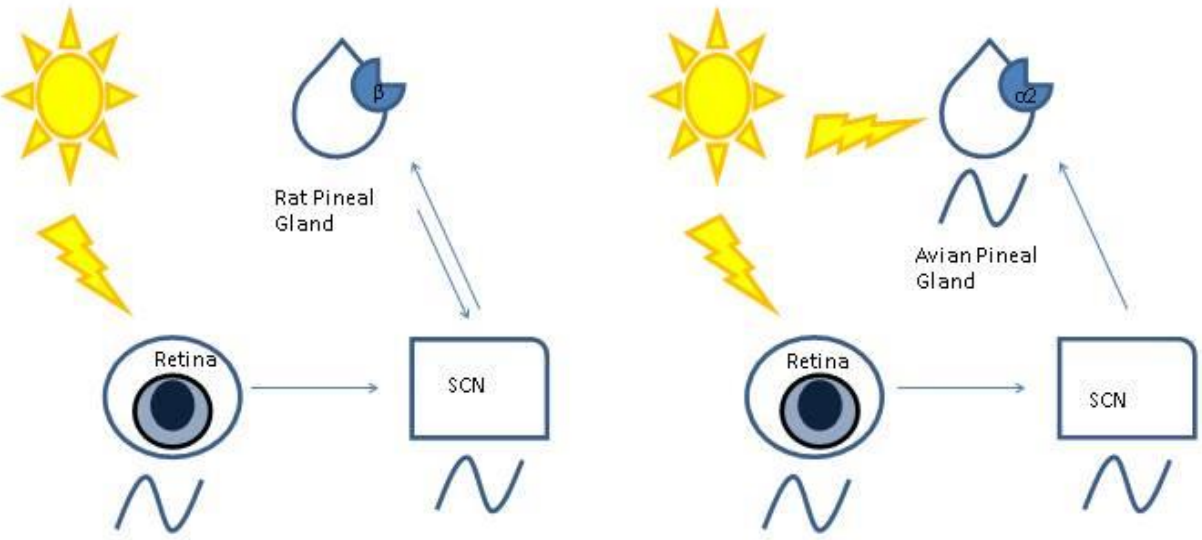

Fig. 5)Representing some differences seen between the mammalian circadian system and the avian circadian system. In rats the information from light passes from the retina to the SCN and then to the pineal gland where melatonin can be produced. The rat pineal gland has β receptors of the stimulatory type.. In birds the light signal reaches the pineal gland directly, as well as thorough the retina. In the chicken the pineal gland, SCN and retina all contain their own circadian oscillators. The chicken pineal gland mainly has α2 inhibitory receptors.. This figure is adapted from a figure published in an article in the Cell Tissue Research in 2002: **Arjun Natesan · L. Geetha · Martin Zatz** Rhythm and soul in the avian pineal.

1.7) Melatonin:

Melatonin is called the “darkness” hormone in vertebrates as it peaks during the night phase both within the pineal gland and plasma, both in diurnal and nocturnal species (Zatz M, 2000) (Zatz, 2002). This simple monoamine hormone is secreted by the pineal gland. Its production is controlled by the circadian clock with a zenith in the dark period. Melatonin, the key messenger hormone of the circadian system is prepared from the amino acid tryptophan in a four step process. The result of the second step is serotonin, a widely available bioactive material in the vertebrate organism (fig.6). Its function is to spread the photoperiodic signal giving the organism information about the day/night cycle as well as reflecting the seasonal changes in daily length (Stehle JH, 2003) (Bailey MJ, 2009) (Korf HW, 2003).

In mammals melatonin acts on two types of highly sensitive receptors, MT1 and MT2, both of which are seven-membrane G-protein coupled receptors (Korf HW, 2003). Melatonin receptors are known to act through the cAMP signalling pathway. The importance of melatonin as a timing cue on peripheral tissues has been shown in experiments done on the pars tuberalis (PT) of mice, which demonstrated that in melatonin proficient mice the *mPER1* peaks right after the drop in melatonin. In contrast in melatonin deficient mice, or mice lacking MT1 receptor the rhythmic expression of *mPER1* is completely abolished (Stehle JH, 2003) (Korf HW, 2003). This shows that endogenous melatonin acts directly on the melatonin receptors in the PT to time the rhythmic expression of clock genes within the PT. Through acting on the MT1 receptor of PT in mice, melatonin can have an effect on the endocrine system. In chicken three melatonin receptors have been identified. Mel1a is a homologe to the mamalian MT1, Mel1b is a homologe to MT2, and Mel1c (Rada S JA, 2006) (Paulose JK, 2009).

The rhythmic release of melatonin can be regulated by many environmental physical stimuli amongst others by light and temperature. Rhythmic changes in the serum levels of melatonin are known to play a key role in the sleep wake cycle and in the balance of serotonergic transmission in the brain. Melatonin has also been shown to be part of the entrainment of the circadian system to both phase advance and phase delay. In mice it has been shown that

endogenous melatonin acts on MT2 receptors within the SCN to facilitate phase shifting of the circadian rhythm in response to a change in the light dark schedule (Pfeffer M, 2012).

An impairment of the rhythmic circadian melatonin release can result in many disorders. It has been shown that there is an increased risk of depression, seasonal affective disorders, night time cluster headaches and advanced sleep phase syndrome etc. if melatonin levels are chronically elevated (Durlach J, 2002). On the other hand, continuously depressed melatonin levels are correlated to an increase in anxiety, daytime migraines, delayed sleep phase syndrome, light induced epilepsy and chronic weakness (Durlach J, 2002).

Studies have also shown that melatonin may be involved in the regulation of gonadal function through acting on the hypothalamic-pituitary-axis. Circadian disruption can lead to decreased levels of melatonin which in turn can lead to increased levels of gonadotropins (Davis S., 2006). This may cause an increase in testosterone or estradiol which may be part of the underlying mechanism of increased risk of cancers such as breast and prostate in people exposed to chronic shift work (Davis S., 2006). Additonally melatonin has been shown to have growth-inhibatory effects, and perhaps even anti-mitotic effects (Davis S., 2006) thus disruption of the melatonin secretion may directly increase the risk of cancer development. Melatonin has even been implemented in the development of diabetes, especially diabetes type two (Kitazawa, 2012). These are only a few examples of the multitude of functions melatonin plays, but they highlight why a better understanding of disruption of melatonin synthesis is important.

The avian pineal gland can generate rhythmic expression of melatonin both under *in vivo* and *in vitro* conditions (Zatz, 2002). In birds the expression of melatonin can be detected already from the second week of embryonic development during *in vitro* conditions (review by Zeman M, 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) concentration within the pineal gland at ED20 (Michal 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 M., 2011) (Zatz M, 2000) (Klein, 2006).

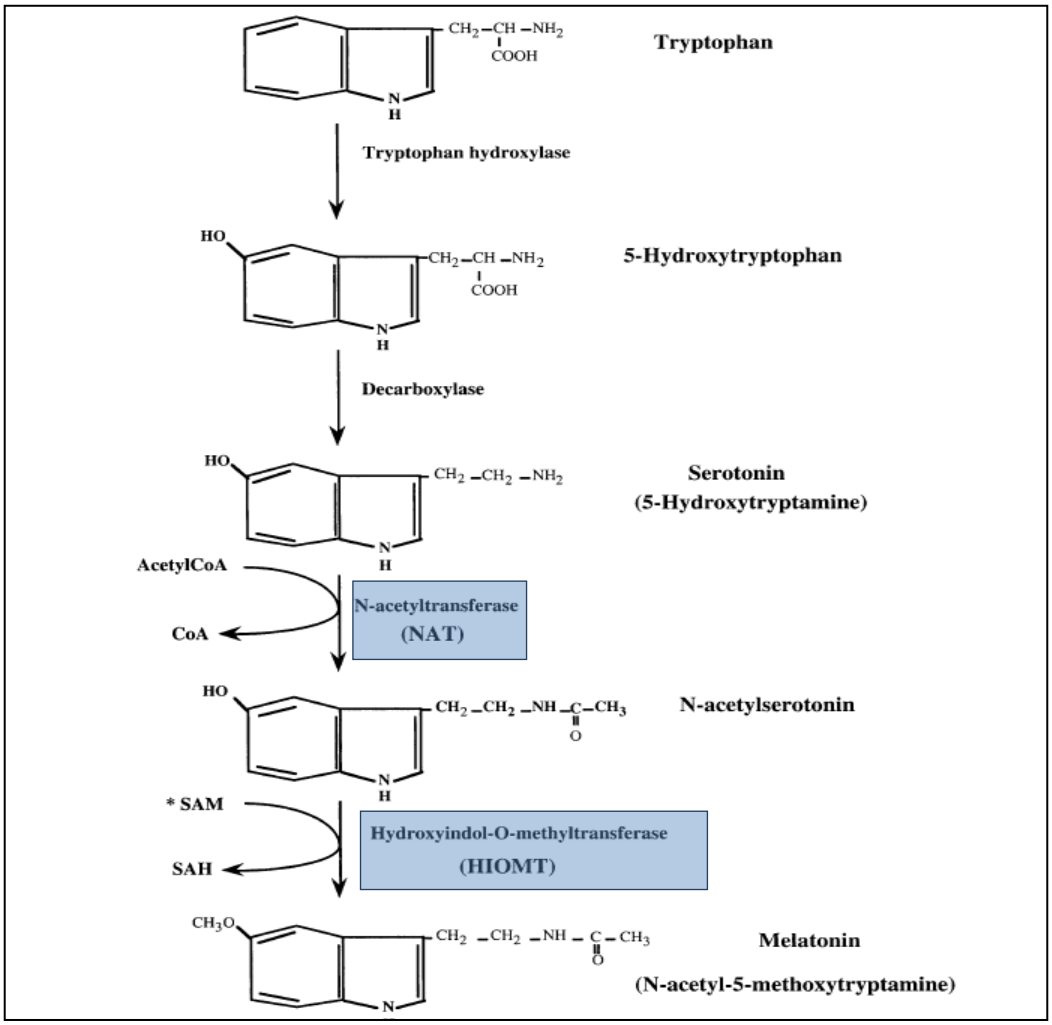

Fig.6) Synthesis of melatonin from tryptophan within the pineal gland. In birds the production of melatonin within the pineal gland directly corresponds to the mRNA expression of *AANAT*.

1.8) Aim of our experiments:

- To study if the transcriptional regulation of the circadian clock gene *clock* shows any response to acute changes in the environmental light-dark conditions both *in vivo* and *in vitro*

- To prove that the embryonic chicken pineal gland is a suitable model for studying circadian rhythm-related disorders at the molecular level by investigating the expression of genes involved in melatonin synthesis such as *AANAT* and hydroxyindole-O-methyltransferase (*HIOMT)* together with the circadian clock gene *clock* under DD, LD, and phase delay of four hours (LD+4h) acute phase shifting conditions

2) Materials and Methods:

2.1. Animals

2.1. a) Experiment with post-hatch chicken pineal glands:

Newly hatched white Leghorn chickens were kept under 14/10 h LD schedule (lights on at 6:00) for 6 weeks. The chickens were illuminated with fluorescent lamps providing 400 lux light intensity measured at the level of the bird's head. Food and water were available ad libitum. In the *in vivo* experiments, chickens were placed at 20:00 into a 10/14 h DL environment (reversed cycle, lights on at 20:00). Chickens were sacrificed by decapitation in 4 h intervals beginning at 18:00, and the pineal glands were collected.

In the *in vitro* experiments post-hatch chickens from the LD group were sacrificed at 12:00 and the pineal glands were placed into a multichannel perifusion system (fig. 4). The experiment ran for 3 days. (Nagy A.D., 2007). The *in vitro* experiments were carried out under LD conditions until the second day at 20:00, when conditions were changed to DL (lights kept switched on from 20:00 until 6:00). Pineal specimens were removed from the perifusion chambers every 4 h, with the first time point being 18:00 on the second day.

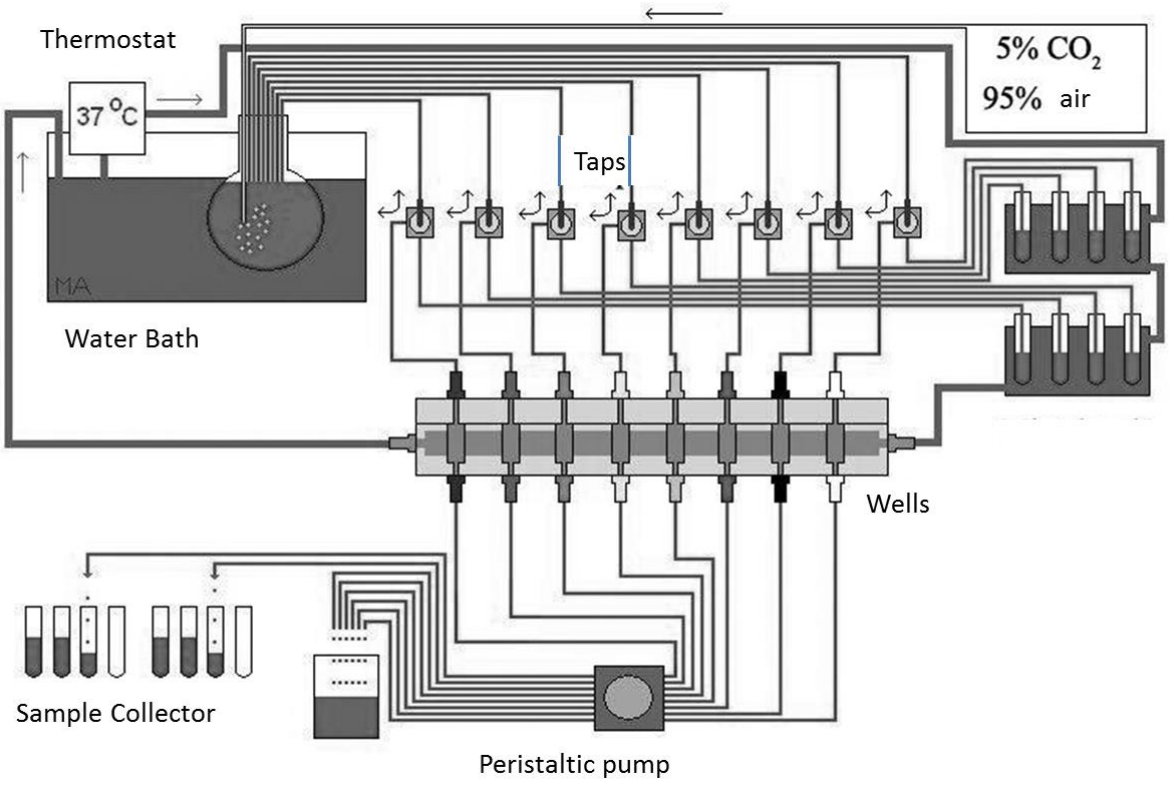

Fig. 4) Schematic representation of the 8 chamber perifusion system. Drawing was done by Attila Matkovits.

2.1. b) Experiments using embryonic chicken pineal glands

Fertilized eggs of white Leghorn chicken were incubated from embryonic day 1 (ED1) at a temperature of 37.5 ^{o}C, and humidity between 60-70%. Egg rocking was done automatically every 2 hours. Control eggs were kept under 12 hour light 12 hour 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 hours (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. Semiquantitative RT-PCR

Total RNA was extracted from pineal glands of post-hatch chicken with Sigma's TRI Reagent following the manufacturer's protocol (T9424, Sigma–Aldrich, St. Louis, Missouri, USA). Absorbance was measured at 260nm with Ultrospec 2100 instrument. Total RNA extracts (200 ng) of each pineal specimen were analyzed with primers for clock mRNA (forward: TCCCAGTCTCTTGGACAACC, reverse: GCTGTTGCTGGATCATGTGT) and b-actin mRNA (forward: GATGGACTCTGGTGATGGTG, reverse: AGGGCTGTGATCTCCTTCTG) as an internal standard. The one-step semi-quantitative RT-PCR was run with 5 U MMLV Reverse Transcriptase (Life Technologies, Carlsbad, California, USA) and 0.2 U RedTaq DNA polymerase (Sigma); this protocol was optimized and standardized previously (Nagy A.D., 2007). The conditions were as follows: RT – 42 ^{o}C 15 min, 94 ^{o} C 5 min; and PCR – 26 cycles of 94 ^{o} C 30 min, 60 ^{o}C 30min, and 72 ^{o}C 1min. End products were separated on agarose gels stained with SYBR Green I. Gels were trans-illuminated with blue light. The fluorescence of PCR products was measured as the mean pixel intensities of the DNA bands on gel pictures (ImageJ software, National Institutes of Health, USA).

2.3. Real-time quantitative RT-PCR.

Total RNA of embryonic chicken pineal glands was extracted as mentioned above. 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 ^{o}C 2 min, 95 ^{o} C 10 min, 40 cycles of 95 ^{o}C 15 sec and 60 ^{o}C 1 min). For quantitation calculations the delta-delta Ct method was used, after control measurements for reaction efficiency normalizations.

2.4. 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) Experiments on post-hatch chicken:

(Published in: Expression pattern of clock under acute phase-delay of the light/dark cycle in the chicken pineal model. – Gen. Comp. Endocrinol. 172: 170-172. 2011.)

3.1. a.) *In vivo* effects of acute inversion of the light/dark schedule on the 24 hour pattern of *clock* expression in the chicken pineal gland

The pineal glands were collected over a 30 hour period every four hours. The first sample was taken 2 hours prior to the shift in the light/dark schedule at 20:00. In the LD group *clock* mRNA levels increased during the dark phase with a peak at 2:00 which corresponds to ZT 20 (ZT 20 indicates the time 20 hours after the lights have been switched on).

The DL group showed a significant increase in the *clock* mRNA levels already at 22:00, merely two hours after the unexpected light exposure at night, which is assumed to be a response to the acute phase delay of the normal LD cycle (fig .7). However during the second DL cycle the levels of *clock* mRNA is significantly decreased at 2:00 during the "new" light phase compared to the control group (fig. 7).

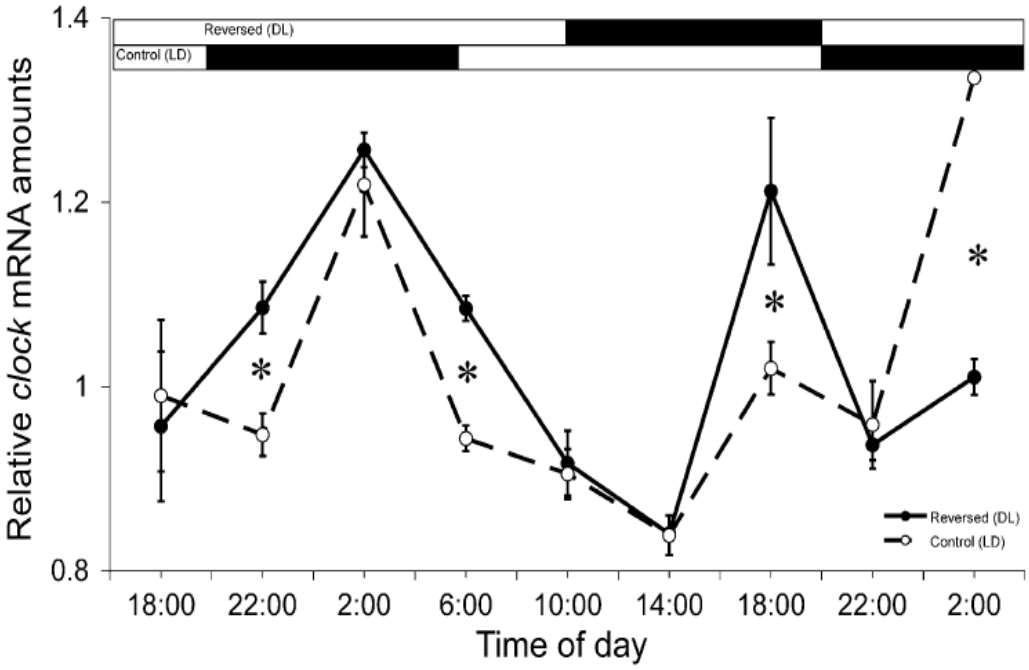

Fig.7) *In vivo* effects of a phase-delayed light/dark schedule on the 24 h pattern of clock mRNA expression in the chicken pineal gland. Graphs represent relative *clock* mRNA levels of pineal glands at each time point (mean of n = 3, ±SEM) collected from chickens exposed to a reversed LD cycle (DL) from 20:00 (—) or kept under LD conditions (- - -) as a control group. Horizontal bars show the light/dark conditions for each group (black indicates darkness). Significant differences ($p < 0.05$) between groups are indicated with *.

3.1. b.) *In vitro* effects of acute inversion of the light/dark schedule on the 24 hour pattern of *clock* expression in the chicken pineal gland

Pineal glands were collected over a 20 hour period every four hours. The first sample was taken 2 hours prior to switching the LD conditions to DL at 20:00. In the LD group the peak of *clock* mRNA was seen at subjective night at 22:00/ZT6 (fig. 8).

In the DL group the levels of *clock* mRNA remained stable during the first cycle, without any significant peaks. There was no significant change in the *clock* mRNA levels between the LD and the DL groups within the first 12 hours of the new DL (fig. 8).

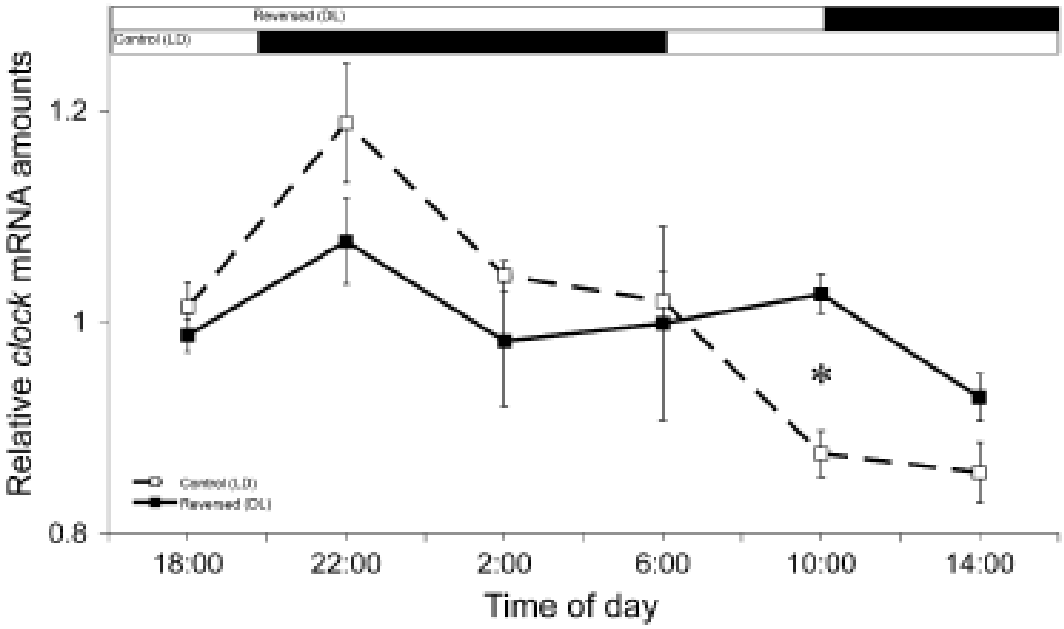

Fig. 8) *In vitro* effects of a phase-delayed light/dark schedule on the 24 h pattern of *clock* mRNA expression in the chicken pineal gland. Graphs represent the relative *clock* mRNA levels of pineal specimens (mean of n= 3, ±SEM) that have been subject to a reversed LD cycle (DL) from 20:00 (-) or kept under normal LD conditions (- -) as a control group. The keys are similar to those in fig. 5. Pineal samples were collected *in vitro* with four hour intervals, with the first sample being taken 2 hours prior to a switch from LD to DL. Significant differences ($p < 0.05$) between groups are indicated with *.

3.2) Experiments on embryonic chicken:

(Currently in press, Gen Comp Endocrinol, 26th CECE Proceedings Volume 2013, original paper.)

3.2. a.) *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 ZT 12, 12 hours prior to the switch in the lighting conditions, every four hours until ED 21. The control group was kept under LD conditions.

AANAT shows a robust daily rhythm, even under DD conditions (fig. 9). Lack of light at ZT 4:00 does not cause a significant change in the expression of *AANAT*. There is no significant difference in the expression of *AANAT* between the LD and DD groups throughout the duration of the experiment.

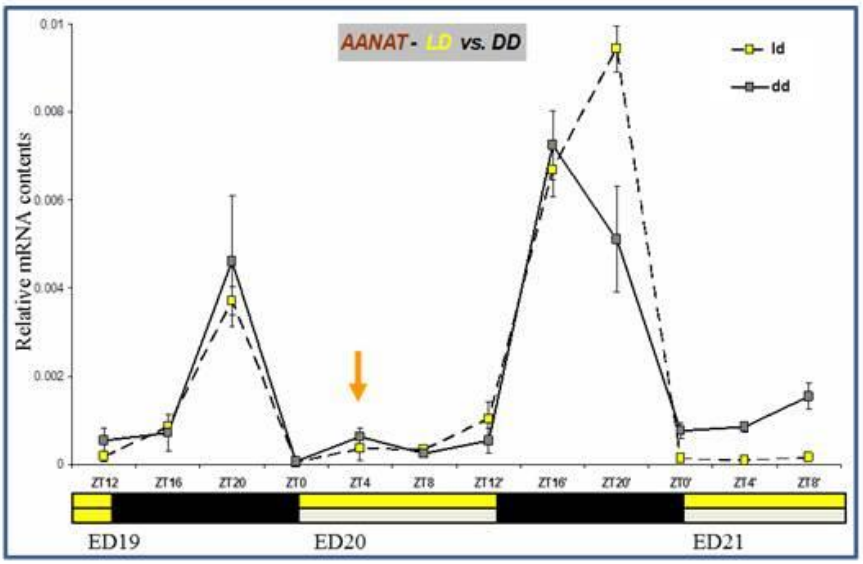

Fig.9) *In vivo* effect of DD compared to LD on the expression of *AANAT* in the embryonic chicken pineal gland. Pineal glands were collected every four hours starting from ED 19 at ZT12. Graphs represent the relative *AANAT* mRNA level of the pineal glands at each time point (mean of n = 3, ±SEM) collected from chicken exposed to DD (-) and the control group LD (- -). Arrow indicates time point of interest.

3.2. b.) *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. 10).

Under DD conditions at ZT4 the absence of light causes a shift in the expression of *HIOMT* compared to that seen under LD (fig. 10). Then in the the second cycle of DD there is a lower expression of *HIOMT* at ZT4, compared that seen under LD conditions.

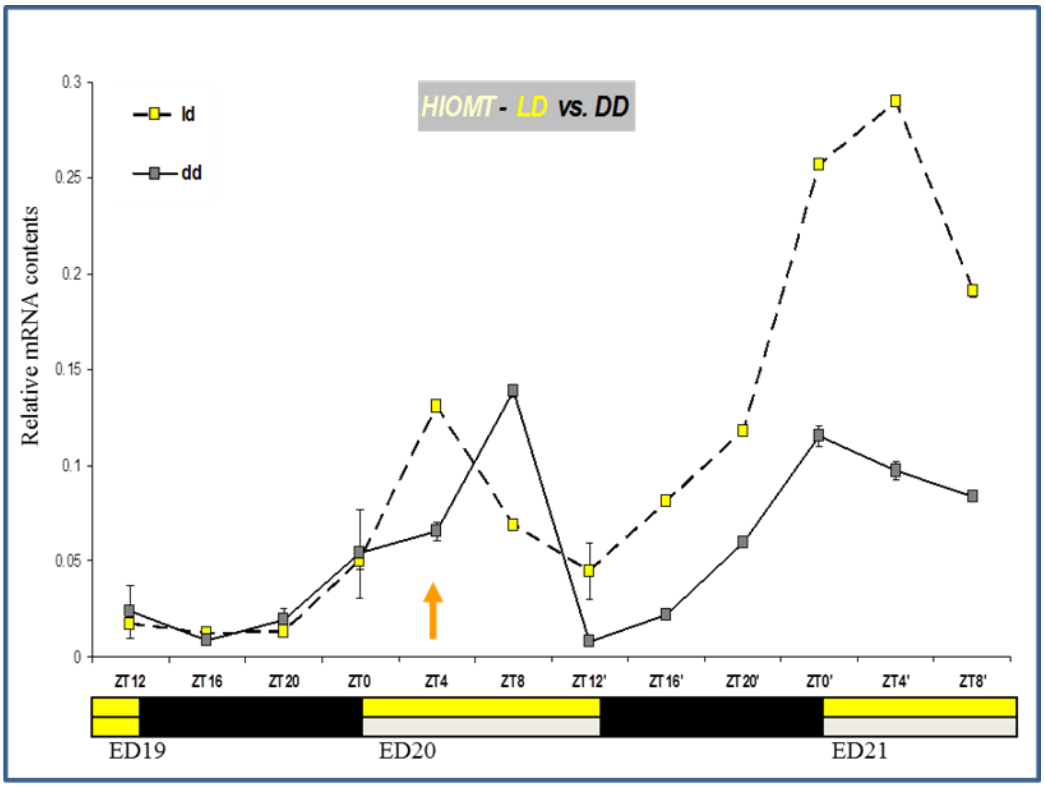

Fig.10) *In vivo* effect of DD compared to LD on the expression of *HIOMT* in the embryonic chicken pineal gland. Pineal glands were collected every four hours starting from ED 19 at ZT12. Graphs represent the relative *HIOMT* mRNA level of the pineal glands at each time point (mean of n = 3, ±SEM) collected from chicken exposed to DD (-) and the control group LD (- -). Arrow indicates time point of interest.

3.2. c.) *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. 11). 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. 11).

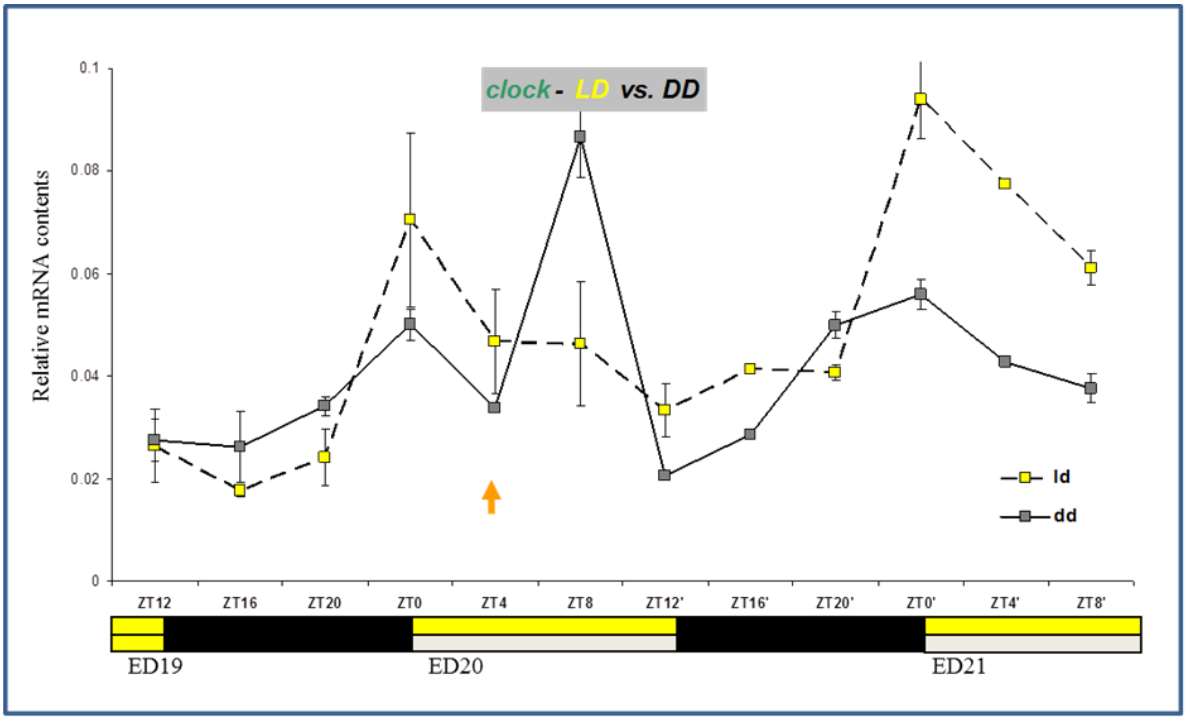

Fig.11) *In vivo* effect of DD compared to normal LD on the expression of *clock* in the embryonic chicken pineal gland. Pineal glands were collected every four hours starting from ED 19 at ZT12. Graphs represent the relative *clock* mRNA level of the pineal gland collected at each time point (mean of n = 3, ±SEM) from chicken exposed to DD (-) and the control group LD (- -). Arrow indicates time point of interest.

3.2. d.) *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 hours 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 hour period.

In the LD group *AANAT* shows a clear robust 24 hour rhythm (fig. 12). 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.

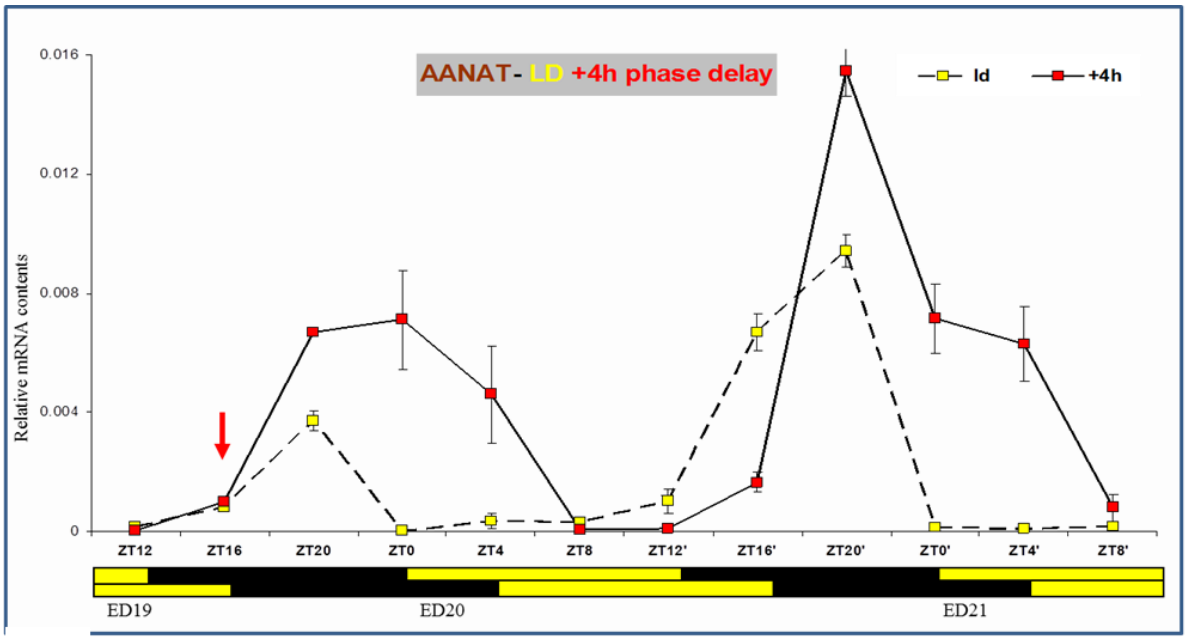

Fig.12) *In vivo* effect of 4 hour phase delay (LD+4) on the expression of *AANAT* in the embryonic chicken pineal gland compared to normal LD . Pineal glands were collected every four hours starting from ED 19 at ZT12, prior to the change in light schedule. Graphs represent the relative *AANAT* mRNA level of the pineal glands at each time point (mean of n = 3, ±SEM) collected from chicken exposed to LD+4 (-) and the control group LD (- -). Arrow indicates time point of interest.

3.2. e.) *In vivo* effect of four hour phase delay on the expression of *HIOMT* in the embryonic chicken pineal gland compared to normal LD conditions:

ED19 eggs were subjected to a four phase delay (LD+4). Pineal glands were collected every four hours, with the first time point being at ZT12, prior to the change in the light schedule.

In the LD group *HIOMT* shows a high amplitude rhythm with a peak during the light phase (fig.13). In the LD+4 group there is no acute change seen in *HIOMT* expression within the first four hours of prolonged light exposure (ZT16) when compared to the LD group. At ZT8 the peak for *HIOMT* expression shows phase delay in the LD+4 group compared with the LD group (fig.13).

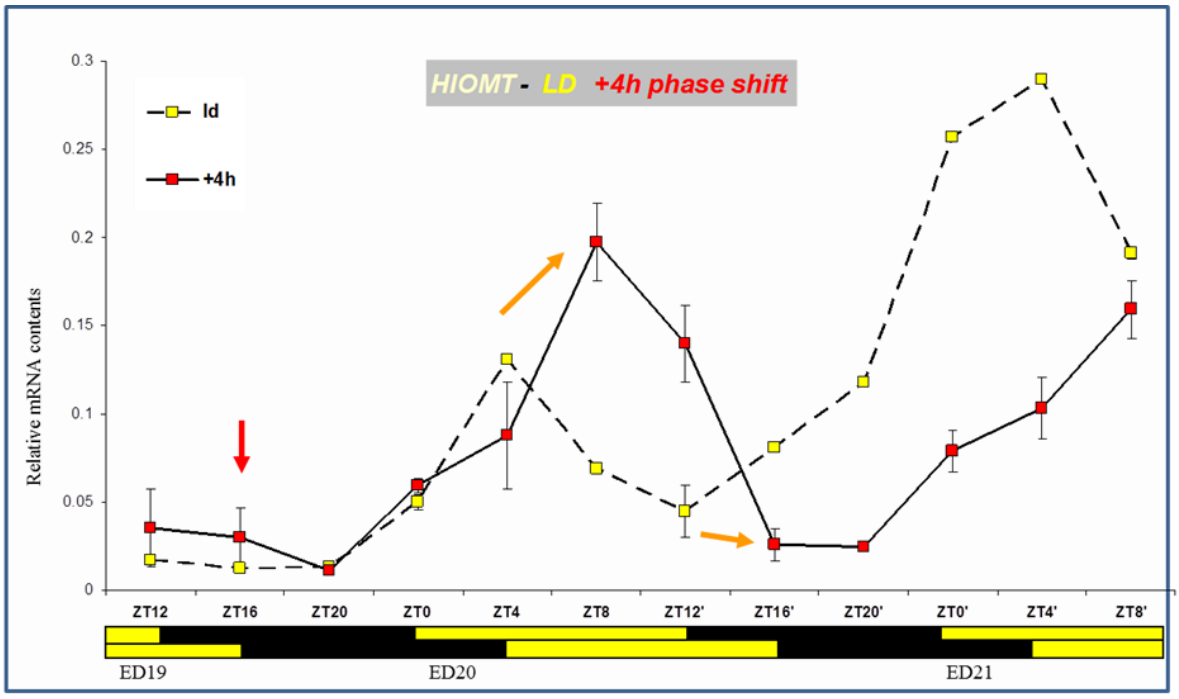

Fig.13) *In vivo* effect of 4 hour phase delay (LD+4) on the expression of *HIOMT* in the embryonic chicken pineal gland compared to normal LD . Pineal glands were collected every four hours starting from ED 19 at ZT12, prior to the change in light schedule. Graphs represent the relative *HIOMT* mRNA level of the pineal glands at each time point (mean of n = 3, ±SEM) collected from chicken exposed to LD+4 (-) and the control group LD (- -). Arrows indicate time points of interest.

3.2. f.) *In vivo* effect of four hour phase delay on the expression of *clock* in the embryonic chicken pineal gland compared to normal LD conditions:

On ED19 eggs were subjected to a 4 hour phase delay (LD+4) of the light schedule. Pineal glands were collected every four hours from ED19-ED21, and the first collection occurred prior to the phase delay.

Under LD conditions *clock* shows a clear 24 hour pattern with a peak during the light phase (fig. 14). Within the first four hours of phase delay at ZT16, *clock* mRNA expression shows acute change with a clear peak in its expression. Furthermore the phase delay of the *clock* mRNA expression is maintained until the end of the experiment, seen clearly by a shift of the rhythm compared to that of the LD control group (fig. 14).

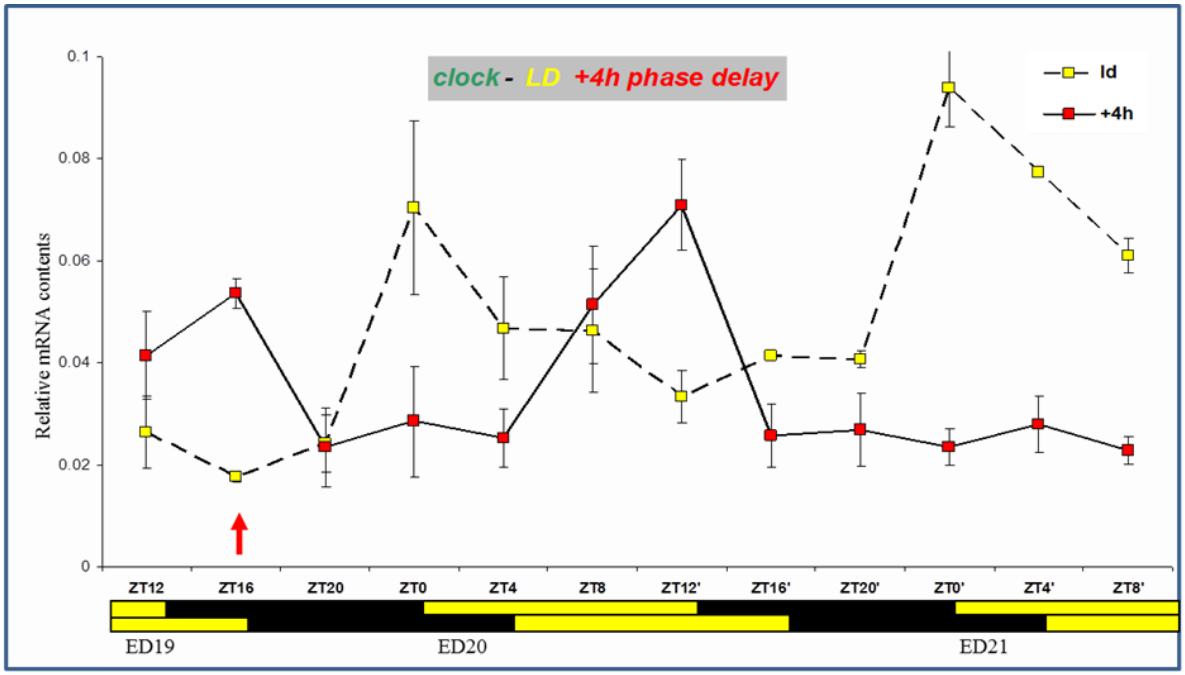

Fig. 14) *In vivo* effect of 4 hour phase delay (LD+4) on the expression of *clock* in the embryonic chicken pineal gland compared to normal LD conditions. Pineal glands were collected every four hours starting from ED 19 at ZT12, prior to the change in light schedule. Graphs represent the relative *clock* mRNA level of the pineal glands at each time point (mean of n = 3, ±SEM) collected from chicken exposed to LD+4 (-) and the control group LD (- -). Arrows indicate time point of interest.

4) Discussion:

In post-hatch chicken kept under normal LD cycles, the *in vivo* (control group) *clock* shows a peak in expression during night time (fig.7). In contrast to some data in the literature which has been published earlier (Helfer G., 2006) (Rajaratnam S.M.W., 2001) (Yoshimura T., 2000), but consistent with others (Bailey M.J., 2003) (Okano T., 2001) (Yasuo S., 2003), our data supports the idea that under normal entrained conditions the transcriptional activity of *clock* in the chicken pineal gland shows daily oscillations, with a maximum during the dark phase under *in vivo* conditions. The oscillation in *clock* mRNA expression is known to have a small amplitude (it was around 1.5 fold in this study), which may explain why others suggested earlier that *clock* is constitutively expressed within the chicken pineal gland (Okano T., 2001) (Toller G.L., 2006) (Chong N.W., 2000). Dispensable oscillations which are too subtle to detect may also play a role (Zheng X., 2008) which is not essential for the rhythm control, similar to what is seen for CLOCK expression in the mammalian SCN (Shearman L.P., 2000).

When the chickens were exposed to a reversed light cycle (DL) *clock* mRNA expression showed an acute change within the second hour of unexpected light exposure *in vivo* (fig. 7). This indicates that there is an acute change in the transcriptional control of *clock* in response to the acute phase delay of the periodic environmental stimuli. Further studies should be carried out to determine the underlying mechanism of this acute change to determine which complexes are involved in the light dependent response of *clock* transcription *in vivo* in the chicken pineal model.

When the chicken pineal glands were exposed to prolonged DL conditions *clock* mRNA levels decreased during the light phase (fig. 7). The difference between the mRNA expression of *clock* during the first cycle of DL and the second cycle of DL conditions may indicate that unexpected light exposure and expected light exposure trigger different transcriptional mechanisms in the chicken pineal gland. For *cry1* it has previously been demonstrated that opposite transcriptional control is involved during the first DL cycle and in the second DL cycle (Nagy A.D.).

In vitro under normal LD conditions *clock* mRNA levels peak during dark phase (fig. 8). This data corresponds to earlier published data which demonstrate that *clock* mRNA shows a similar rhythm with peaks during dark phase both *in vivo* and *in vitro* in the chicken pineal gland (Bailey M.J., 2003) (Karaganis S.P., 2008) (Okano T., 2001).

After placing the pineal glands under DL condition *in vitro,* the expression of *clock* mRNA showed no significant peak in the first cycle, and likewise in the second cycle, there was no significant difference when compared to the control LD group. This suggests that *clock*, unlike *per2* (Yoshimura T., 2000), is not directly induced by light which is similar to that seen in the mammalian SCN (Shearman L.P. M. Z., 1999).

The clear difference seen in the expression of *clock* mRNA under DL *in vivo* (fig. 7), where there was a clear difference between the control and experimental group, and *in vitro* (fig. 8) where there was no clear difference between the DL and LD group, may indicate that there are also neuroendocrine signals involved in the regulation of *clock* in response to acute changes in the environmental lighting conditions (Nagy A.D., 2007).

The development of the circadian rhythm has been in focus of circadian experiments for a long time. The presence of both clock genes and genes involved in the production of melatonin has been demonstrated during ontogenesis in various species (Ribelayaga C, 1998) (Jimenez-Jorge S, 2007) (Martin-Robes AJ, 2010) (Nagy A.D. S. K., 2008) (Goncalves L, 2012) (Akasaka, 1995). In our experiments done on embryonic chicken pineal glands, *AANAT,* a key enzyme involved in the synthesis of melatonin shows a robust rhythm with a peak during the dark phase (fig. 9). This observation is in concordance with previously published data (Toller G.L., 2006) (Bernard M G. J.-C., 1999). The change of the light schedule from LD to DD did not cause a significant change in the expression of *AANAT* (fig. 9), as expected. There was a lack of both acute changes to sudden darkness and a lack of a delayed response seen in the second cycle of DD. This supports the idea that *AANAT* in the embryonic chicken pineal model is regulated primarily by the pineal clock itself, which corresponds to previously published data (Haque R, 2010), as opposed to being a light sensitive gene.

Exposing the embryonic chicken to a phase-delay of 4 hours (LD+4), essentially prolonging lights on by four additional hours, did not cause an acute change in the expression of *AANAT* (fig.12). The second cycle of LD+4 caused a phase delay of the expression of *AANAT*; the whole rhythm of *AANAT* mRNA expression was shifted compared to that of the control LD group (fig.12). This again suggests that *AANAT* is not directly light sensitive as it did not respond acutely to prolonged light exposure, but that in fact it is regulated by the circadian clock itself.

HIOMT is the other key enzyme of melatonin production which has also been demonstrated to be present both in the pineal gland and in the retina during development (Jimenez-Jorge S, 2007). In our experiments on embryonic chicken pineal gland under normal LD conditions the expression of *HIOMT* mRNA shows a high amplitude rhythm with a peak during the light phase (fig. 10), which corresponds to data published earlier (Bernard M, 1993). Once the chicken were placed under DD the expression of *HIOMT* mRNA was shifted compared to that seen in the control group (fig. 10). During the second cycle of DD there is a significant difference seen in the expression of *HIOMT* when compared with the LD group. There is a damping of the rhythm amplitude of *HIOMT* under DD, but it still shows a peak during the light phase and a general 24 hour pattern which again correlates with data earlier published (Bernard M, 1993) (Bernard M G. J.-C., 1999).

The exposure of the embryonic chicken to a 4 hour phase delay did not cause any acute changes in the expression of *HIOMT* mRNA (fig. 13). However during the second cycle of LD+4 there is a clear phase delay of the expression of *HIOMT*, indicating that also this gene is regulated by the circadian clock.

The presence of a rhythmic expression of both *AANAT* and *HIOMT* under LD, DD and LD+4 in this experimental protocol proves that the chicken embryonic clock at ED19-21 has a functioning clock mechanism, and that it is a good model for phase shifting experiments as well as experiments on the ontogenesis of the circadian clock in the chicken pineal gland.

Clock is a clock gene acting as a positive regulator together with *Bmal1* and *Npas2*. Amongst its functions it acts together with *Bmal1* as a heterodimer to regulate the expression of *AANAT* (Haque R, 2010). In fact knockdown of clock has been shown to cause a decrease of *AANAT* activity, as well as *Npas2* and *Per2* demonstrating that this is an essential component of the circadian clock (Haque R, 2010). Clock has been demonstrated to be present during development in amongst others fish (Martin-Robes AJ, 2010) and chicken (Goncalves L, 2012).

Placing the embryonic chicken under DD did not cause any acute changes in the expression of *clock* mRNA during the first four hours (fig. 11), when compared to the LD group. However 8 hours later there is a peak in the expression of *clock* which indicates some role for *clock* in light-dependent synchronization *in vivo*. In the second cycle of DD the expression of *clock* was damped, but it still maintained a rhythmic pattern. This correlates well with the results seen for *AANAT* which did not show a change in its rhythm under DD compared to LD.

In the embryonic pineal glands exposed to a four hour phase delay (LD+4) there was a two-fold change in the rhythm of *clock* mRNA expression. First of all there was an acute change seen within the first four hours of prolonged light exposure. *Clock* expression showed a clear peak in at ZT16, which was absent under LD conditions (fig. 14). Secondly *clock* mRNA expression showed a clear phase delay of its rhythm in response to LD+4 conditions which persisted until the end of the experiment. This again indicates a role for *clock* in light-dependent synchronization *in vivo*.

To summarize the embryonic chicken experiments we can clearly see that *AANAT*, *HIOMT* and *clock* all show clear 24 hour rhythms within the embryonic pineal gland, and rhythms which in fact resemble those seen in entrained adult animals (Bernard M G. J., 1993) (Bernard M G. J.-C., 1999) making this an excellent model for further circadian experiments. Furthermore it is clear from the LD+4 experiments that one cycle of altered lighting conditions is sufficient to alter the expression of clock genes themselves as well as genes involved in melatonin synthesis making this a good model to carry out future phase-shift experiments.

5) Conclusion:

Our studies have demonstrated that the effects of change of the light dark schedule on clock genes and clock controlled genes is varied depending on the type of change and the duration of the change. An acute change in the lighting schedule may lead to an acute change in clock genes which are light sensitive, such as *clock*, and only a delayed change in genes which are clock controlled such as *AANAT* and *HIOMT*.

In addition we have proven that the embryonic chicken pineal gland is an adequate model to do circadian experiments, including phase shift experiments. The embryonic pineal gland clearly shows a rhythmic expression of certain clock genes such as *clock* and also of clock controlled genes such as *AANAT* and *HIOMT*. Additionally the embryonic pineal gland is light sensitive and responds to changes in the light dark schedule in a similar manner to the adult chicken pineal gland.

Further experiments should be done to determine the underlying mechanisms of the different transcriptional regulation of clock genes and clock controlled genes that operate during the first and the second cycle of altered light dark conditions.

6) Summary of New Results:

- In adult chicken *clock* shows and acute change to a reversed light cycle (DL) already within two hours of unexpected light exposure *in vivo.*
- Under prolonged DL conditions the expression of *clock* in adult chicken is decreased. This suggests that there are different transcriptional regulatory mechanisms during the first and second cycle of DL exposure.
- *In vitro* DL conditions had no effects on the expression of *clock* in adult chicken.
- *AANAT* expression was not affected by DD conditions in the embryonic chicken pineal gland.
- The first cycle of LD+4 had no effect on the expression of *AANAT*, however during the second cycle there was a phase delay in the expression of *AANAT*.
- The expression of *HIOMT* was damped under DD conditions in the embryonic chicken.
- *HIOMT* expression was not affected by LD+4 during the first cycle of LD+4. During the second cycle there was a clear phase delay of *HIOMT*.
- *Clock* showed now acute changes to DD conditions in the embryonic chicken pineal gland.
- In response to LD+4 *clock* showed a new peak at ZT16, and additionally a phase delay of its rhythm.

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8) Publications:

Peer reviewed articles related to this thesis:

1. S. Kommedal, G. Bódis, A. Matkovits, V. Csernus and A. D. Nagy: Expression pattern of clock under acute phase-delay of the light/dark cycle in the chicken pineal model. – Gen. Comp. Endocrinol. 172: 170-172. 2011. [IF: **3.267**]
2. S. Kommedal, V Csernus, AD Nagy.: The embryonic pineal gland of the chicken as a model for experimental jet lag - Gen Comp Endocrinol *in press* 2013 [IF-2011: 3.267]

Other peer reviewed articles:

3. A. D. Nagy, S. Kommedal, K. Seomangal and V. Csernus: Circadian Expression of clock genes clock and cry1 in the embryonic chicken pineal gland. - Annals N.Y. Acad. Sci. 1163: 484-487. 2009. [**IF: 2.303**]
4. A. D. Nagy, K. Seomangal, S. Kommedal and V. Csernus: Expression of cry2 in the chicken pineal gland: effects of changes in the light/dark conditions. - Annals N.Y. Acad. Sci. 1163: 488-490. 2009. [**IF: 2.303**]
5. Lengyel Zs., Lovig Cs, Kommedal S, Keszthelyi R, Szekeres Gy, Battyáni Z, Csernus V, Nagy AD. Altered expression patterns of clock gene mRNAs and clock proteins in human skin tumors. Tumour Biol. 2013 Apr;34(2):811-9. [IF-2011: 2.143]

Conference Abstracts

1. Nagy A. D., Kommedal S., Csernus V.: Effects of PACAP exposure on the pineal clock gene expression in birds. - Clin Neurosci/Ideggy Szle 60 (S1). 47. 2007.
2. Nagy A.D., Kommedal S., Csernus V.: PACAP hatása az óragén-expresszió cirkadián ritmusára csirke tobozmirigyben. - Magyar Idegtudományi Társaság 12. Kongresszusa, Szeged, 2007. január 26-29.
3. Nagy A.D., Kommedal S., Seomangal K., Csernus V.: Az óragén expresszió cirkadián ritmusának kialakulása csirke tobozmirigyben. - XIV. Sejt- és Fejlődésbiológiai Napok, Balatonfüred, 2007. április 15-17.
4. Kommedal S., Seomangal K., Nagy A.D., Csernus V.: An insight into the development of the circadian clock in the chicken pineal model - Magyar Élettani Társaság Vándorgyűlése, Pécs, 2007. június 8-11. (**poster**)
5. Seomangal K., Kommedal S., Nagy A.D., Csernus V.: Expression of Cry2 in the chicken pineal gland: Effects of changes in the light/dark conditions. - Magyar Élettani Társaság Vándorgyűlése, Pécs, 2007. június 8-11.
6. Nagy A.D., Kommedal S., Seomangal K., Matkovits A., Csernus V.: Expression of clock genes in the embryonic chicken pineal gland. – 11th Biennial Meeting of the Society for Research on Biological Rhythms, Destin, Florida, USA. May 17-22. 2008.
7. Kommedal S., Seomangal K., Nagy A.D**.,** Csernus V.: Expression of Cry2 in the chicken pineal gland: Effects of changes in the light/dark conditions. – 24th Conference of the European Comparative Endocrinologists, Genoa, Italy, Sept 1-6. 2008. (**poster**)
8. Nagy A.D**.,** Kommedal S., Seomangal K., Matkovits A., Csernus V.: Expression of clock genes in the embryonic chicken pineal gland. – 24th Conference of the European Comparative Endocrinologists, Genoa, Italy, Sept 1-6. 2008.
9. Nagy A.D**.,** Kommedal S., Butenschön V., Bódis G., Csernus V.: The pineal oscillators in chicken embryos need no LD cycles to start. – XIth Conference of the European Biological Rhythms Society, Strasbourg, France, Aug 22-28. 2009.
10. Kommedal S., Bódis G., Matkovits A., Nagy A.D**.,** Csernus V.: An insight into the development of the circadian clock in the chicken pineal model – 25th Conference of the European Comparative Endocrinologists, Pécs, Hungary, Aug 31-Sept 4. 2010. **(oral)**

11. Bódis G., Kommedal S., Matkovits A., Nagy A.D.**,** Csernus V.: Clock mRNA expression patterns in the chick pineal gland under experimental jet lag – 25th Conference of the European Comparative Endocrinologists, Pécs, Hungary, Aug 31-Sept 4. 2010.

12. Lengyel Zs., Kommedal S. (presenting author), Lovig Cs., Battyáni Z., Keszthelyi R., Szekeres Gy., Csernus V., Nagy A.D.: Expression of circadian clock genes per1, per2, clock, and cry1 in human melanoma skin biopsies. 13th Biennial Meeting of the Society for Research on Biological Rhythms, Destin, Florida, USA. May 19-24. 2012. **(poster)**

13. Lengyel Zs., Kommedal S., Lovig Cs., Battyáni Z., Keszthelyi R., Szekeres Gy., Csernus V., Nagy A.D.: Expression of circadian clock genes per1, per2, clock, and cry1 in human melanoma skin biopsies. 26th Conference of the European Comparative Endocrinologists, Zürich, Switzerland, Aug 21-25. 2012.

14. Kommedal S., Lengyel Zs., Mattkovitcs A, Csernus V., Nagy A.D.: The Embryonic Chicken Pineal Gland as a Model for Experimental Jet Lag 26th Conference of the European Comparative Endocrinologists, Zürich, Switzerland, Aug 21-25. 2012. **(Oral)**

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