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Function of circadian clocks in peripheral tissues

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"We know circadian clock genes are out there; but what are they doing?" Riede 2009

Preface

In this thesis I will review and reflect the present knowledge on functional aspects of clock genes in the peripheral tissues of the mammalian body. 'Peripheral' in this thesis can be interpreted in it's broadest context; all oscillating tissues outside the suprachiasmatic nuclei (SCN).

The SCN are seen as the central, or 'master' clock in mammals. It is located in the ventral hypothalamus above the optic chiasm, and consists of a small number of endogenously oscillating cells. Rhythms generated by molecular feedback loops comprising several clock genes and their transcripts. It's phase ('time') can be set by light-input from the photosensitive cells in the retina, but also many other external and internal factors are known to affect this clock in one way or another. In turn the SCN influences a great extent of tissues and body functions, both in the brain and in the rest of the body, by a number of output pathways. The SCN is thought to be essential for the synchronization of the body in the timing of all it's behavioural and physiological processes.

In the last decades several studies have suggested or shown the importance of circadian rhythms in gene expression in tissues outside the SCN, in this thesis referred to as 'peripheral oscillations'. The goal of this thesis is to map our current knowledge on the functional aspect of these peripheral oscillators. Including by which mechanisms they are influenced, how they interact with other timed processes (synchronization) and how they function in vivo.

Abstract

Life on this planet has evolved elaborate mechanisms to anticipate on predictable environmental changes in night and day caused by the earths axial rotation, thereby maximizing fitness. In mammals a broad variety of behavioural and physiological processes correlate to the phase of the day, sleep/activity patterns as their most obvious example. These circadian rhythms are more then just direct responses to environmental cues (as light) and remain present under constant conditions, showing intrinsically generated oscillations.

The SCN, two hypothalamic nuclei, are seen as the master clock; synchronizing many peripheral processes to each other. The SCN receive direct light input from the retina as their strongest 'time cue', and are able to adjust the intrinsic clock to the matching environmental 'time'. These intrinsic oscillations, in clock gene expression and neuron firing can be observed in SCN tissue both in vivo and vitro. Also isolated peripheral tissues, both neuronal and non-neuronal, show oscillations and rhythmic gene expression, but these are often dampened without SCN synchronization input. This shows the importance of a central pacemaker.

In recent years some studies found evidence for SCN independent peripheral oscillations in mammals. The current knowledge of their role and functioning will be addressed in this thesis. We focus on circadian gene expression rhythms in the retina, liver, and testes, as well as the brain regions involved in memory and stimulus assessment. This thesis will show circadian rhythms are widely expressed throughout the body, and often depend on both local oscillations and central input. Also the circadian clock genes expressed in the SCN might have different functions and interactions in peripheral tissues compared to their function in the central clock. In all it shows the importance of local circadian organization in our physiology and it shows our limited knowledge on the topic of peripheral oscillators.

Samenvatting (Dutch summary)

In het leven op aarde zijn uitgebreide biologische mechanismen geëvolueerd om te kunnen anticiperen op de voorspelbare veranderingen van dag en nacht, voortkomend uit de rotatie en baan om de zon van onze aarde. Hiermee wordt overleving geoptimaliseerd. In zoogdieren is een breed scala aan gedrag and fysiologische processen gecorreleerd aan de fase van de dag-nacht cyclus, slaap-waak ritmes als duidelijkste voorbeeld. Deze circadiane ritmes zijn meer dan enkel directe reacties op veranderingen in de omgeving (zoals lichtintensiteit) en blijven ook in constante condities aanwezig, wat wijst op een intrinsiek karakter van deze cyclische veranderingen.

De SCN, twee nuclei in de hypothalamus, worden gezien als de hoofdklok; en zorgen dat veel perifere processen qua timing op elkaar afgesteld worden. De SCN ontvangen directe informatie over licht vanuit de retina, wat als belangrijkste 'tijdssignaal' gezien wordt, en zijn in staat de interne klok hierop aan te passen. De intrinsieke oscillaties van de klok in de SCN, kunnen zowel *in vivo* als *in vitro* worden geobserveerd via neuron activiteit en in gen expressie. Ook geïsoleerde perifere weefsels, zowel neuronaal als niet neuronaal, vertonen oscillaties over de dag. Echter zijn deze vaak aflopend in amplitude waarneer SCN synchronisatie input ontbreekt. Dit laat het belang van een centrale pacemaker zien.

In de laatste jaren hebben enkele studies bewijzen gevonden dat er ook SCN onafhankelijke oscillaties in zoogdieren voorkomen. In deze thesis zal de huidige kennis over de rol en functie van circadiane ritmiek buiten de SCN aan de orde komen. We focussen daarbij op circadiane genexpressie ritmes in de retina, lever en testes in zoogdieren. Daarnaast komen ook hersengebieden betrokken bij geheugen en associaties in deze thesis naar voren. Deze thesis laat zien dat circadiane ritmes overal in het lichaam voor kunnen komen, en zowel onder SCN als onder locale regulatie kunnen staan. Ook kunnen de circadiane klok-genen binnen en buiten de SCN verschillende functies en interacties vertonen. In totaal laat deze thesis zien dat locale circadiane organisatie essentieel is voor onze fysiologie en tevens laat het zien hoe weinig we nog maar van perifere oscillatoren afweten.

Introduction

The earths rotation around the sun and it's own axis produces predictable temporal changes in the environment of all living organisms on our planet. It provides us with the differences of night and day in temperature rhythms and light intensity. Not only these 24-h cycles, but also seasonal and tidal rhythms with differing cycle-lengths are direct effects of earths orbit. In the 3.5 billion years life has been evolving on our planet, organisms have developed specialised mechanisms to monitor these rhythms and anticipate to the cycle. By these 'timing' or 'clock' mechanisms organisms are able to anticipate to future events, maximising fitness by better survival and reproductive succes of the induvidual.

Although clock-mechanisms are widely distributed throughout all forms of life, the field of science specialising on understanding how these processes work only 'just' emerged. Chronobiology, as this field is called, has become a blooming discipline and gained increased interest in the last century. Integrating biological, ethological, genetical, molecular, neurobiological and physiology-based principles with 'time'- or timed processes, chronobiology focuses on the temporal rhythms exhibited by life forms all around us.

Perhaps the most dominant rhythms observed in and around us are those related to the phase of the day/night cycle; rhythms with a period length of close to 24h; hence the term *circadian* (circa = *about / dian= a day).* These rhythms are driven by internal clock mechanisms. In order to truly deserve the term 'clock', these time mechanisms all share a very important feature; they are able to oscillate on their own, in the absence of external cycles. This principle was first already discovered in the 18th century by the French astronomer De Marian, who observed that cyclic leave movements in plants remained present when the plant was transferred to a room derived of light. This showed that diurnal rhythms are more then just a direct response to light; the system responsible was able to generate rhythmicity on it's own. De Marian proved by his experiment the first criterium of a circadian rhythm; it must be endogenously generated.

Biological rhythms have two other criteria; they must be insensitive to temperature, and maintain a stable period length under broad range of temperatures. This excludes biochemical and enzyme mediated processes. Third is the ability to be set or adjust (synchronised) to a certain phase of the day by external stimuli.

Currently biological rhythms have been shown in a broad range of processes, tissues and organisms, ranging from growth and spore formation in fungi, like *Neurospora crassa* [Bianchi DE, 1964], to body temperature variations in mammals as ourselves [Wever RA, 1986]. The expanding amount of research on chronobiology has revealed many of our behaviour and physical functioning is dominated, guided, controlled or related to our circadian 'clock' mechanisms.

The focus in this thesis is on the mammalian clock system, comprising the latest insights on the interplay of the various clock-genes both in the central clock and peripheral (in this thesis, peripheral will mean outside the SCN clock). We adress recent findings on the function of circadian clock gene expression for individual tissues, and how these genes are involved in the optimalisation of physiological processes and guiding behavioural. Not all processes are linked to the same phase of the day, giving rise to an intricate network of feedback and feed forward loops of gene transcription patterns, with many differences in role and function depending on the cell type in which they are expressed. Synchronization of multiple cells, multiple tissues and even different organs is essential to optimize our complex behaviour and physiology. The assumption of one central 'master clock', to orchestrate all rhythms in our bodies, seems very plausible.

First we shall give a short introduction on the basic principles of chronobiology. Important is the search for the location of the bodies central clock, a puzzle which seemed to be solved by the discovery of two paired hypothalamic nuclei in the mammalian brain; called the *suprachiasmatic nuclei.*

The SCN as the master clock

These suprachiasmatic nuclei are located in the ventral hypothalamus, on top of the optic chiasm and directly below the base of the third ventricle. In 1972, Stephan and Zucker found that when they

lesioned this specific brain area in rats the animals became arrhythmic in their sleep-wake behaviour [Stephan FK and Zucker I, 1972]. What followed was a string of research on many behaviour and physiological parameters which lost their circadian pattern when the SCN region was destructed. Rhythmicity in hormonal levels of renin, insulin, corticosterone, TSH, prolactin and ACTH were shown to abolish when the SCN was lesioned [Abe K *et al.*, 1979; Pan JT and Gala RR, 1985; Stoynev A *et al.*, 1980; Szafarczyk A *et al.*, 1981]. Since then, the list of processes in the mammalian body depending on the SCN for temporal organization has grown exponentially. Not only hormones, but also hearth rate and blood pressure [Janssen BJ *et al.*, 1994] are shown to have a circadian component. The SCN was responsible for, or involved in, virtually all rhythmicity in our bodies, so it seemed.

The clock machinery

When the era of molecular genetics emerged by the development DNA sequencing techniques [Sanger F *et al.*, 1977] and the polymerase chain reaction (PCR) technique developed by Mullis in 1983 [Saiki RK *et al.*, 1986] a whole new level of approaching biological processes became possible. Not soon after there development, these techniques were used in chronobiology as well. Mutant strains of fruit flies, D*rosophila melanogaster*, with unusually long and short behavioural rhythms were sequenced to determine the genes responsible. This lead to the discovery of the genes *period*^{S} and *period*^{L}, genetic sites in which an altered genetic sequence caused unusually short or long activity cycles [Reddy P *et al.*, 1984]. After the identification of these first 'circadian clock genes' in insects, homologues were found in many different organisms, including mammals.

The basics of our current understanding on the molecular clock system in mammals, and which genes are involved is nicely described in the review article of Panda and Hogenesch in Nature [Panda S *et al.*, 2002]. A heterodimer of two transcription factors, *CLOCK* and *BMAL-1* bind to the E-box regions of *Period* (*Per1,Per2* and *Per3*), *Cryptochrome* (*Cry1* and *Cry2*) and nuclear receptor (*REV-ERBα* and *RORα)* gene promoters which leads to their transcription. *Per* and *Cry* proteins heterodimerize, and act in the nucleus as transcription factors themselves. The *Per/Cry complex* then represses the activity of the *CLOCK/BMAL-1* complex thereby negatively affecting their own transcription in turn. The nuclear receptors *REV-ERBα* and *RORα* bind to sites in the BMAL-1 promoter, regulating the transcription of *BMAL-1*. Additional factors like kinases, present in the cytoplasm, effect protein degradation.

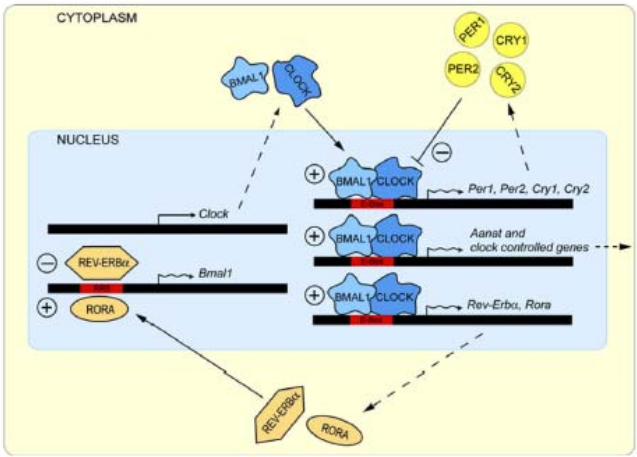


Figure 1; Interplay of core clock genes in mammals. The mammalian molecular feedback loops, comprizing the positive loop in which *BMAL1* and CLOCK proteins dimerise to form a transcription complex that facilitates transcription of *period,cryptochrome*, nuclear receptors *Rev-Erba* and *Rora* and clock controlled genes. The transcription of Bmal is inhibited by PER:CRY complexes (the first negative loop) and can also be regulated by the nuclear receptors. Figure adopted from (*Tosini G et al., 2008)*

Because there is a delay in time between the binding to a promoter in the nucleus and the translation of the proteins in the cytoplasm, an oscillation of all these factors is the net result. Besides these 'core-feedback loops' there are many other factors that influence these process. For instance light input from the retinal-hypothalamic-tract (RHT) is known to effect *CREB*-elements in *period* promoters, effecting transcription as well. Additionally, proteins from this core clock gene loops can affect transcription of other proteins, like AANAT which will be discussed later, giving the clock many different pathways to translate into functional rhythmic processes in the cell. So this basic loop is effected by input factors on this molecular clockwork, and the clockwork itself is connected the a high number of output mediators, which can be very different between tissues [Bozek K *et al.*, 2009; Ko CH and Takahashi JS, 2006; Panda S *et al.*, 2002].

The molecular clockwork, described above, is not only present in the SCN, but in probably almost all cells of the body. Several studies have shown these clockwork proteins oscillate in peripheral tissues and individual cell types as well, like in the liver, lungs, adipocytes, and leukocytes to name a few [Bando H *et al.*, 2007; Fukuya H *et al.*, 2007; Gomez-Santos C *et al.*, 2009]. But showing rhythmicity in the transcription of these core clock genes itself doesn't provide us with any understanding of their role and function. The aim of this thesis is to couple peripheral clock gene rhythms to functional processes.

Setting the central clock in the SCN by light

Light is the strongest zeitgeber in mammals; meaning that light is the most important cue about the phase of the day to our internal 'clock-time'. The SCN as we discussed before receives light input from the retinohypothalamic track (RHT). The retina translate the light dark signal to the SCN via the RHT by glutamate and a protein called PACAP. It is important that the SCN–phase corresponds with the phase of the day/night cycle (accuracy of the clock). Therefore the clock can be advanced or delayed by light input, to match the right environmental time. In nocturnal mammals an phase advance is induced when light is received in the late subjective night. and when light is received in early subjective night it produces a phase delay of the molecular clock-gene loops in the SCN. In the previous section we mentioned the CREB-elements (Ca^{2+}/cAMP response binding-element protein) in the *Period* promoters. Phosphorylation of these sites results in either an advance or a delay in the transcription of *Period* genes, thereby effecting the whole oscillation of the clock-gene loops. PACAP and glutamate are capable of phosphorylating these sites in sensitivity windows which in turn are affected by the phase of the endogenous clock [Hannibal J *et al.*, 2001; Hannibal J *et al.*, 2008; von Gall C. *et al.*, 1998].

The SCN synchronizes peripheral clocks by melatonin

Although the SCN affect many behavioural and physiological rhythms both direct and indirect the melatonin rhythm is especially important to discuss before moving on the peripheral oscillators.

Melatonin levels are high during night, and low during the day in both diurnal and nocturnal mammals. Melatonin is synthesized in the retina and the pineal gland. In the retina it acts as a local mediator in light sensitivity. The melatonin synthesised in the pineal has more extended peripheral effects. Melatonin is synthesized from it's precursor tryptophan by several enzymes, including arylalkylamine N-transferase (AANAT). Left, this figure adopted from Falcon et al. shows this biosynthesis pathway [Falcon J *et al.*, 2009]. The melatonin rhythms rely mainly of the rhythmic presence of AANAT, both in the pineal and in the retina [Klein DC, 2007]. AANAT transcription is directly coupled to the core clock genes; the CLOCK/BMAL complex binding to the E-Box in the promoter of this gene. The SCN signals to the pineal in the subjective night by norepinephrin, which lead to a increase in intracellular Ca^{2+} and cAMP levels in the pinealocyte all leading to an increase in AANAT activity [Falcon J *et al.*, 2009; Klein DC, 2007; Tosini G *et al.*, 2007].

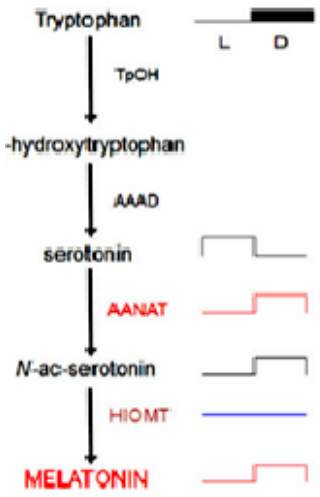


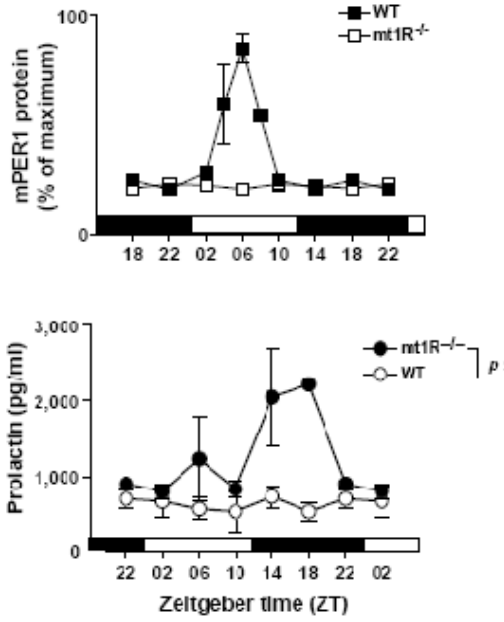
Figure 2; Melatonin biosynthesis pathway. The right panel (top) shows the light dark cycle. The rate limiting enzyme in melatonin synthesis AANAT (arylalkylamine N-Acetyltransferase) is expressed during the dark, driving melatonin synthesis in turn. HIOMT and TpOH, the other enzymes in melatonin synthesis are expressed in constant levels over the circadian day. (Figure adopted from Falcon J et al., 2009)

Melatonin has been shown to effect many central and peripheral tissues and plays multiple roles. Maybe the most important finding is that melatonin has profound effects in the *pars tuberalis* of the adenohypophysis [Stehle JH *et al.*, 2003; von Gall C. *et al.*, 2002b]. This region, in the basal hypothalamus and just above the pituitary, is very dense in melatonin receptors and also an key component in the endocrine system. The pars tuberalis passes input from the thalamus (wich in turn is connected to many brain structures guiding behaviour appetite, sleep and more), to the pituitary. The

pituitary transduces this input to output by releasing hormones and other humoral factors. Many peripheral tissues respond in turn these hormones. Also the pituitary itself expresses melatonin recptors.

Both in *pars tuberalis* tissue and the pituitary circadian gene expression our found to be dependant on melatonin.In hamsters with pinealectomy, the rhythm in clock genes in the *pars tuberalis* diminishes [von Gall C. *et al.*, 2002a]. , showing the importance of melatonin in synchronising many physiological processes in the mammalian body.

By now we have discussed the essentials in understanding the central clock in mammals. The three components of this central clock system (light reception; clock genes rhythms and melatonin output) are situated in mammals in separate tissues, but are in fact evolved from structures that combined these features in one tissue. This is still seen in many non mammalian vertebrates. Now we can take the next step in describing peripheral clocks in mammals; how they are interacting with these central clock properties and how they function in translating clock-gene cycles to physiological processes.



Melatonin is important to the HPA axis. Top picture shows mPER1 protein levels in the pars tuberalis. In melatonin receptor type 1 KO mice the peak in PER1 expression during the day is absent. In melatonin receptor KO mice prolactin is severely overexpressed in the pituitary. Melatonin has profound effects on the mammalian endocrine system [von Gall C. et al., 2002].

Peripheral Oscillators

Although the central clock in the SCN is important to sustain and synchronize rhythms in many peripheral tissues, the last decades many researchers have found rhythmicity in isolated tissues and cells as well. The mammalian circadian timing system is not located in the brain alone, but functions more like a body-wide web of oscillators which have both an endogenous component and are influenced by the central oscillator of the SCN. Individual SCN cells have been shown to generate an autonomous ~24h rhythms in firing rate for many days [Liu C and Reppert SM, 2000]. Also the SCN tissue expresses continues rhythmicity in clock gene expression when isolated. Figure 3a,b,c; (adapted from Yamazaki *et al.*) shows rhythms in isolated cultured SCN (a), liver (b) and lung (c) tissues by a luciferase construct coupled to the *per1* promoter [Yamazaki S *et al.*, 2000].

The rhythmicity in the SCN (a) remains present for multiple weeks, suggesting it to be highly autonomous and endogenously regulated. The rhythms in peripheral tissues (like here liver and lung) loose without synchronizing input their rhythmicity. After a resetting stimulus (medium change, in lung with serum in new medium) the rhythm in these peripheral tissues reappears, suggesting the absence of the rhythm isn't caused by tissue death. This demonstrates the peripheral clock oscillations are endogenously present in peripheral tissues but are reliant on synchronization signals to function [Liu C and Reppert SM, 2000; Reppert SM and Weaver DR, 2002; Yamazaki S *et al.*, 2000].

There are thus big differences between the central and peripheral oscillations in clock-genes. But why are these molecular clockworks present in so many (if not; all) tissues? We will discuss the role and function of some clock genes in peripheral tissues, mostly recent findings and far from complete understanding.

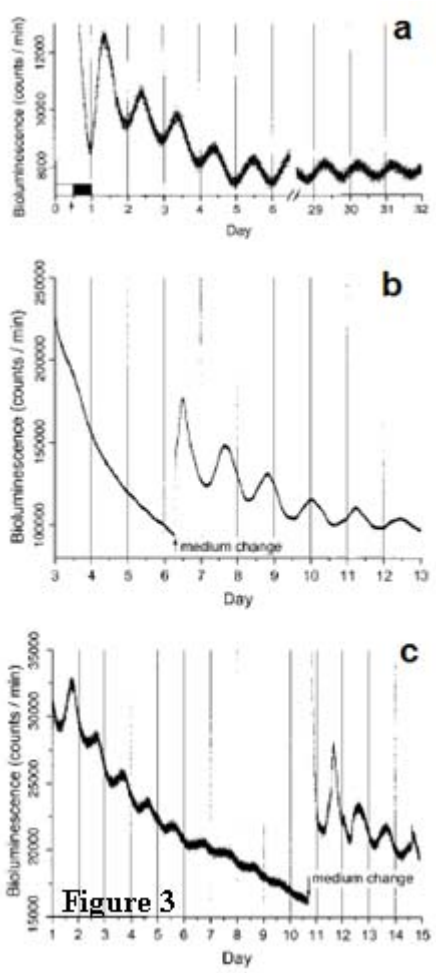


Figure 3; Oscillations in *Per* expression in *Vitro*. Oscillations in *period* gene transcription were observed by coupling the *Per1* promotor to a luciferase construct. In the SCN (a) *Per* is cyclicly produced with an intrisic cycle length of around 24h. These rhythmic expression patterns continue for weeks without any input to the tissue. In peripheral tissues like the liver (b) and lung (c) also circadian *Per* expression is observed with periods of around a day, but these quickly fade away, showing unlike the SCN they are not selfsustaining. A medium change can restore rhythmicity, showing this unability the retain oscillations is not caused by tissue damage. (figure adopted from Yamazaki S et al., 2000)

The clock in the Retina

An highly studied peripheral tissue, in relation with clock-gene expression, is the mammalian retina. We discussed the importance of the retina in synchronizing the central clock of the SCN to environmental light-cycles before. Already in 1996 Tosini and Menaker, showed a clear oscillation in melatonin synthesis in cultured retina of the Syrian golden hamster. Also they noted this cylcle could be entrained to the light dark cycle *in vitro*, and had an intrinsic free running rhythm close to 24h in constant darkness. In the tau mutant hamster, a well known strain with a gene mutation that causes an unusual short intrinsic cycle length (denoted as τ ; pronounced "tau") of ~20h, this retinal free running rhythm in melatonin release was also shorter. This experiments demonstrated a endogenous circadian oscillator was present in the mammalian retina [Tosini G and Menaker M, 1996].

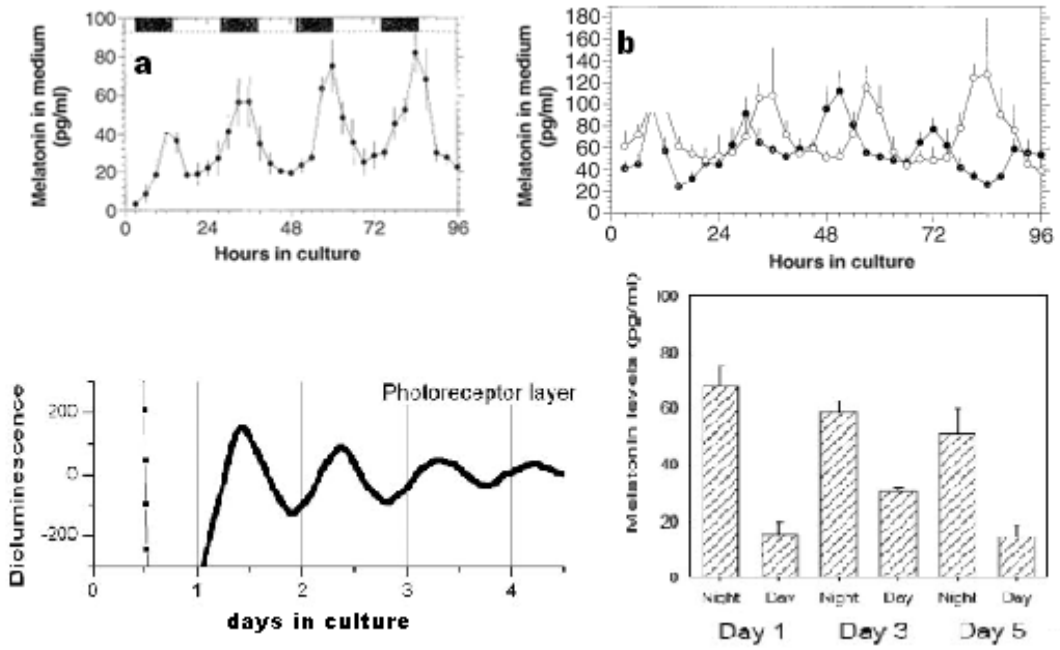


Figure 4; Rhythms in the cultered retina. Figures a shows a circadian rhythm in melatonin release in culterd hamster retina wich can be entrained to light dark cycles. The free running rhythms of wild type hamsters (white symbols) show around 1 cycle each 24h, whereas the retina of tau mutant hamsters produce melatonin in free running cycles of close to 20h (figures a and b adopted from Tosini G and Menaker M, 1996). Bottom panels; the isolated photoreceptor layer (PRL) of the retina shows a circadain rhythm in *per1*transcription as shown by an luciferase-construct. The PRL also shows daily rhythms in melatonin synthesis in vitro (lower right). Lower panels adopted from Tosini G et al., 2007.

How the genetic clockwork in the retina is composed and functions was shown by several studies. Another study by Tosini in 2007 analysed the retinal tissues by PCR techniques to assess which of the core clock genes were expressed rhythmically in the retina's photoreceptor layer and in the retina in total. They found all the circadian core genes (*CLOCK, BMAL, Per1,2,3, Cry1,*2 and the nuclear receptors *REV-ERBα* and *RORα)* were expressed in the intact retina. However when they tested the rhythmicity, by a luciferase-*per1* promoter construct, they observed most retina were arrhythmic in their *Per1* expression. When looking at the photoreceptor layer (PRL) however; there was a strong circadian rhythm in *Per1* present (figure 4c). Also all the core clock genes, except *per2* were expressed in this specific part of the retin [Tosini G *et al.*, 2007]. Cultured PRL cells generate cyclic patterns in melatonin synthesis (figure 4 c). These studies demonstrate the presence of an autonomous clock in the PRL of the mammalian retina, driving rhythms in melatonin synthesis, which modulates in turn the sensitivity to light [Tosini G *et al.*, 2007]. These findings suggested the retinal clock lies in the photoreceptor layer of the retina.

The biological importance of having a functioning clock in the retina can best be shown by looking at animals that lack this functional retinal clock. Many strains of knock out mice are available which lack specific components of the core clock gene loops, making the clock-system less effective or even impairing it in total. Mice lacking the BMAL gene in the retina alone, show different electroretinograms (ERGs) compared to control littermates without this induced mutation in the retina (figure 5a,b; adopted from [Storch KF *et al.*, 2007]). Also the rhythmicity in light-processing, shown by ERG responses, is lost in *cry1,2* double knock out mice (figure 5c,d; adopted from [Cameron MA *et al.*, 2008]).

An ERG (figure 5) shows the electrical changes in the eye, caused by a flash stimulus. The first negative deflection represents the activation (depolarisation) of photoreceptors (a-wave), followed by the activation (and depolarisation) of subsequent neurons of the light transduction pathway giving a positive deflection (b-wave). The retina of nocturnal species, like mice, contains so few cones that the are incapable of driving a measurable a-wave. The amplitude of the b-wave depends on the time of the day and is driven by endogenous clocks in the retina. Also the ERG form depends on the type of stimulus (cone or rod activating intensities). We here discuss an cone activating stimulus, so with an high intensity. Normally the b-wave amplitude after an cone activating stimulus is higher in the subjective day compared to subjective night (figure 5 a,c controls; 6CT represents mid day, 18CT represents mid-night). At night mammalian vision is more dependant on rod photoreceptors.

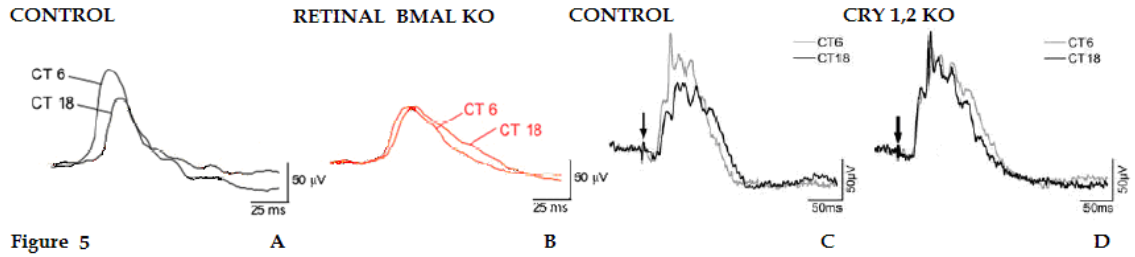


Figure 5; Clock gene KO mice show impairments in circadian rhythms of cone lightperception. Photooptic responses during midday (CT6) and midnight (C18) for (a,c) wild type mice, (b)mice without retinal *BMAL1* expression and (d) *cry1*$^{-/-}$*cry2*$^{-/-}$double knock out mice. Electroretinograms of a cone activating flash stimulus against rod saturating background lighting conditions. The normal circadian regulation would increase cone sensitifity during the day (cone vision) and decrease it during the night (rod vision). This circadian modulation is absent and *Cry1,2*$^{-/-}$ and *Bmal1*$^{-/-}$ mice which have either persistant high cone responses (*Cry*$^{-/-}$) or persistant low cone responses (*BMAL*$^{-/-}$) during subjective night and day. (Figures adopted from Cameron MA et al., 2008; Storch KF et al., 2007)

The ERG studies reflect the responses to bright light stimuli, because they are inflicted by a cone activating flash against a rod saturating background (rods are more sensitive to dim light). BMAL knock out mice show no increase in b-wave in their subjective day, so the circadian rhythm in ERG is lost and b-wave remains at low amplitude. Cameron *et al.* studied the same in *cry* knock out mice, and found the ERG rhythmicity was lost as well. Interestingly however they observed the ERG was similar to the high b-wave of subjective day, so the decrease in the night is absent [Cameron MA *et al.*, 2008]. These opposite effects show 1) disrupting the clock results in al loss of rhythmicity in 2) different clock genes effect different states.

The interpretation of these results might be as follows; the retina comprises both rods (sensitive to low light intensities) and cones (for high light intensities). The results of both Cameron and Storch were done, specifically on cone responses, because the flash was presented against a rod saturating background [Cameron MA *et al.*, 2008; Storch KF *et al.*, 2007]. With light intensities high during the day and low during the night, the retina has an switch in sensitivity. This sensitivity can be mediated by the (de-)coupling of horizontal cells [Wiechmann AF *et al.*, 1988], (changes in light transduction) or by changes at receptor level [Wiechmann AF and Summers JA, 2008]. During the day the cone specific pathway is better equipped for vision, because light intensities are high. So when a cone activating light stimulus is presented during subjective day this signal is readily passed along by secondary neurons, giving a high b-wave amplitude [Emser W *et al.*, 1993]. The opposite is seen in subjective night. Because light intensities are low during the night, cone receptors are less equipped for vision.

Rods operate better at low intensities so more secondary (processing) neurons are connected to rods. When a cone activating flash stimulus is presented, fewer secondary neurons pass this specific signal, giving a b-wave of lower amplitude. Circadian genes are likely involved in the switch between cone (day) or rod (night) oriented vision. This switch is very rigid; only one of the two cells types can be used. When rods are 'on', during nights with low light intensities, the rods block te hyperpolarisation of the cone photoreceptor and rods interact with bipolar cells only. The oposite happens when the 'cone system' is on. The circadian system, showed to be essental in shifting between rod and cone based vison. The precize intracellular pathways by which clock genes can assert this switch are currently unknown however.

The switch between night (rod) and day(cone) vision might be mediated by retinal melatonin output. Melatonin administration (outside retina) is shown to have a negative effect on b-wave amplitude to a cone activating light stimulus [Emser W *et al.*, 1993; Peters JL and Cassone VM, 2005]. Melatonin receptor over expression in the retina enhanced rod light sensitivity and increased both the a and b-wave during subjective night. [Wiechmann AF *et al.*, 1988; Wiechmann AF and Summers JA, 2008].

The clock in the liver

The SCN long was thought to be the main circadian oscillator in mammals. It drives rhythms in many behaviours like the sleep/wake cycle [Ko CH and Takahashi JS, 2006]. Already in 1977 it was shown however that the SCN is not the only oscillator in mammals; also food intake could generate rhythmicity in mammals. Krieger found when food and water was only presented to nocturnal animals (rats) during the day, rhythms in body temperature and plasma corticoid levels shifted to the day phase. When the animals were SCN lesioned these rhythms persisted, suggesting the food driven oscillator lies outside the SCN [Krieger DT *et al.*, 1977]. The exact nature and locus of this second circadian oscillator remained illusive however for many years.

The liver is a key organ in metabolism, more specific in maintaining blood glucose within the desired physiological range. Postprandial plasma glucose levels rise and can be lowered to the desired range by the either the uptake of glucose in muscles, or by storage in the liver in the form of glycogen. During fasting the stored glycogen in the liver can be converted back to glucose. Glucose is then transported out of the liver hepatocytes, lowering blood glucose levels again. Since the liver is such a key organ in metabolism the hypothesis emerged that the liver might be the seat of this second 'food driven' circadian oscillator. A circadian rhythm in glucose release from the liver, independent from feeding time, was shown by several studies [Kalsbeek A and Strubbe JH, 1998; Van Cauter E *et al.*, 1997].

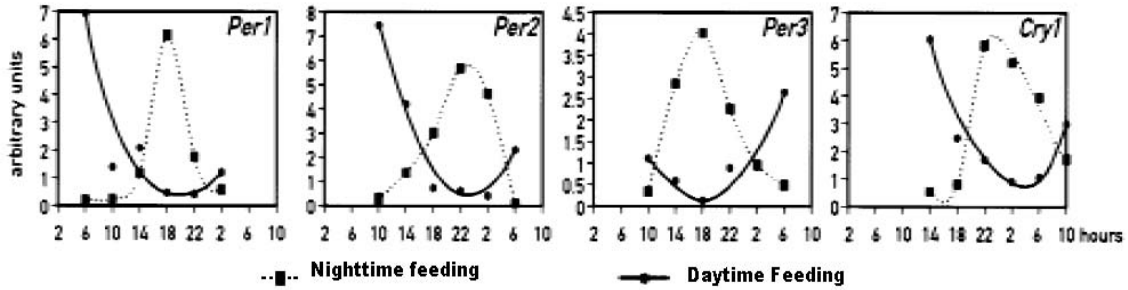


Figure 6; Timing of food affects the phase of clock gene expression in the liver. Mice were housed under 12:12 LD and had only food available during the dark (dottet lines) or light. Animals were held on this food protocol for 8 consecutive days after which they were sacrificed at 4h intervals, to prepare whole cell RNA from the liver. mRNA levels were measured by ribonuclease protection assay, and data plotted. Daytime (sleepphase) feeding caused opsite clock gene rhythms in the liver.

Genetic techniques again helped providing new insights about the mechanisms involved. Food restriction can change rhythms in circadian gene transcription in peripheral tissues while leaving the expression of clock genes in the SCN unaltered [Damiola F *et al.*, 2000]. Figure 6, adopted from Damiola's article, shows the rhythms of several clock genes in the liver can be changed in phase by daytime feeding. The liver shows strong circadian rhythms in gene expression of many genes, beside

the core clock genes. In a genetic analysis by Storch *et al.,* around 10% of all genes expressed in the liver showed a circadian rhythm [Storch KF *et al.*, 2002].

Like in the retina two things were shown to simultaneously happen; rhythms in gene expression and rhythms in physiological parameters. Lamia *et al.* studied the importance of a functioning liver clock by making a strain of liver specific BMAL1 knock out mice (L-BMAL1$^{-/-}$) [Lamia KA *et al.*, 2008].

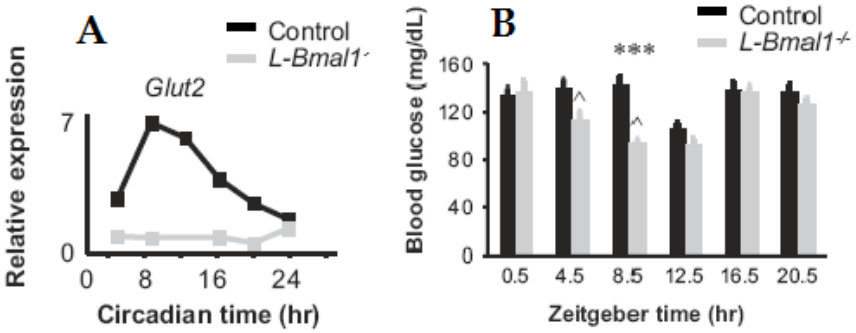


Figure 7

They found rhythmicity in the circadian clock genes in the liver was lost in L-BMAL1$^{-/-}$ mice. Next, they also analysed liver enzymes known to express circadian rhythmicity; including the glucose transporter type 2 (*GLUT2)* protein [Lamia KA *et al.*, 2008].

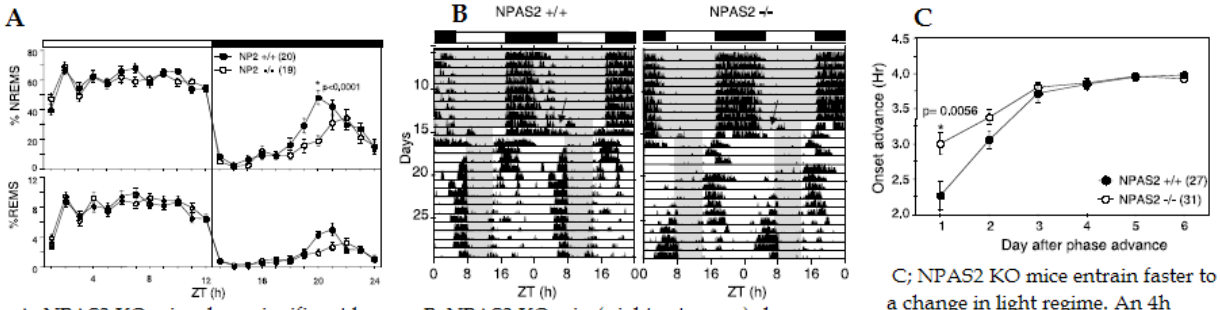
The *GLUT2* transporter is essential in maintaining plasma glucose homeostasis because the liver is the only glucose source during fasting and this transporter is involved in glucose export by the liver. Normally this transporter is expressed more in the fasting state, during subjective day. In L-BMAL1$^{-/-}$ mice this increased expression during the subjective day is absent, causing an impairment is glucose release during fasting. This manifests itself by hypoglycaemia during the subjective day (figure 7b, ZT 0-12 is subjective day). Also clock$^{-/-}$ mice show impairments in maintaining blood glucose levels [Kennaway DJ *et al.*, 2007]. Lamia showed in that in L-BMAL1$^{-/-}$ mice enzymes like glucokinase, liver-pyruvate kinase and adenylate kinase 4 showed loss of circadian rhythmicity as well. This suggests glycogen synthesis, glycolysis and intracellular signalling pathways respectively might be also dependent on intact clock mechanisms, either by direct or indirect effects of clock genes [Lamia KA *et al.*, 2008]. These results show a functioning liver clock is necessary for maintaining sufficient glucose in the fasting state of the day.

The clock in food entrainment

Clock genes interact by special dimerization zones, called PAS domains. Pas domains are important in maintaining an environmentally entrained oscillator [Elvin M *et al.*, 2005], and are present on *Period, Clock* and *BMAL1*. [Looby P and Loudon AS, 2005]. In the mammalian forebrain, more specific the processing areas of sensory inputs and certain emotions like fear and anxiety, an transcription factor containing PAS region, appropriately called neural PAS protein 2 (NPAS2) was found [Garcia JA *et al.*, 2000]. This transcription factor shares functional and sequence homology with the circadian *Clock* gene [Hogenesch JB *et al.*, 1997; King DP *et al.*, 1997]. Like it's orthologue *Clock, NPAS2* can also form a transcription complex by binding with BMAL1 and regulated gene transcription of for instance *Period* genes [Reick M *et al.*, 2001]. Special to NPAS2 might be that it can be influenced by humeral factors like hormones, suggesting a way by which peripheral tissues can be entrained. *NPAS2* might be the peripheral equivalent of *Clock* [McNamara P *et al.*, 2001].

By studies on *NPAS2*$^{-/-}$ mice it is shown that it might be critical in entrainment to non-light stimuli, and it plays major roles in the regulation of sleep and adaptations in behaviour to food restriction protocols [Reick M *et al.*, 2001]. When looking at the distribution of activity in mice, they normally show two peaks in activity, at dusk and dawn. During the middle of the subjective night most mice express break in activity and sleep. *NPAS2* KO mice don not show this interuption in activity and show significantly less sleep during their active (dark) period (figure 10a). Furthermore when subjected to a restricted feeding protocol, in which food is only presented for a small amount of time and outside the normal activity phase, NPAS2 KO mice take longer to entrain. This can been observed in the knock out animals by the absence of food anticipatory behaviour for the first week after introduction to the feeding regimen (figure 10b). However when subjected to a change in light regime

NPAS2 KO mice adapt quicker to new settings (figure 10c) [Dudley CA *et al.*, 2003] (figures also adopted from Dudley *et al.).*



A; NPAS2 KO mice show significant less sleeping behaviour in the active (night) phase of the day.

B; NPAS2 KO mice(right actogram) show impaired food entrainment. Gray background in this figure shows the presence of food.

C; NPAS2 KO mice entrain faster to a change in light regime. An 4h phase advance was induced by a shift in lights on - lights off.

Figure 10

These data suggest there is a forebrain clock with can be entrained by feeding. It might be that *Clock* is involved in light entrainment, whereas NPAS2 is involved in food entrainment. In mice without NPAS entrainment to food is slower, and light entrained behaviour enhanced [Dudley CA *et al.*, 2003]. Additional *Clock* KO mice retain circadian rhythms in the SCN, suggesting NPAS and Clock can take over their orthologues function [DeBruyne JP *et al.*, 2007]. To test the hypothesis *Clock* and *NPAS2* represent light and food driven entrainment pathways, we should find opposite effects in ability to light and food entrainment in *Clock* KO mice. Indeed *Clock* mutant mice retain their ability to entrain to food, show difficulties in light entrainment, and are arrhythmic in constant dark when under food *ad lib* conditions [Pitts S *et al.*, 2003]. NPAS2 might thus be the food entrainment clock gene, whereas *Clock* is more involved in light entrainment. Additional research in the transcription of NPAS in peripheral tissues and the SCN under food restriction protocols might provide interesting new insights about food entrainment.

The clock and memory

Also in memory formation there is an involvement of circadian clock gene expression, mediated by *NPAS2*. NPAS2 KO mice showed impaired cued and conditional learning, and disturbed long term memory [Garcia JA *et al.*, 2000]. Memory formation in animals depends on time place associations. Time place association connect a stimulus (either positive like the presence of food, or negative like danger) to a certain place and time [Cain SW *et al.*, 2004; Van der Zee EA *et al.*, 2008].

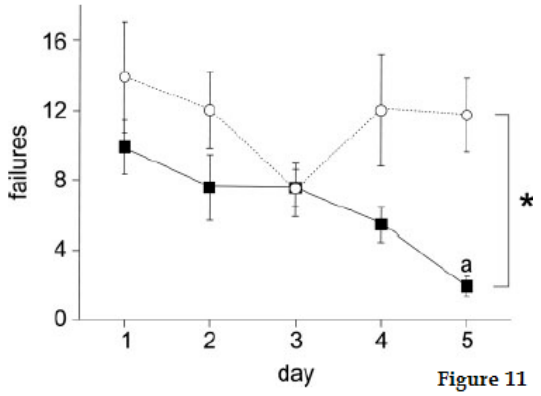


Figure 11

Hippocampal dependent spatial learning task performance of WT (dark) and Per1 KO mice. In an 8 armed maze food was presented at the same location for a number of days. Per1 showed no decline in number of errors (wrong arm entries) wheras from day 3 onward the WT mice 'learned' were to find the food.

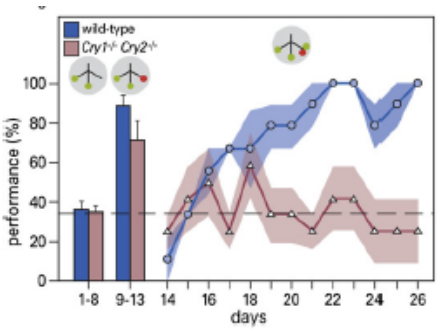


Figure 12;

Time place learning in Cry KO mice, depending on the time of day (morning, mid-day or evening session) a food shock was present in one of three arms. Whereas wild type control mice (blue) learned to which arm to avoid at a specific time, Cry KO (red) mice kept performing on change level (dotted line).

The hippocampus has also been show to exhibit clear circadian rhythms in gene expression [Jilg A *et al.*, 2009]. The peak times in the expression of *Period* genes coincides with the time windows in which memory consolidation occurs. Importantly *Per1* KO mice show an impairment in hippocampus dependend learning tasks (figure 11; adopted from Jilg *et al.*) [Jilg A *et al.*, 2009].

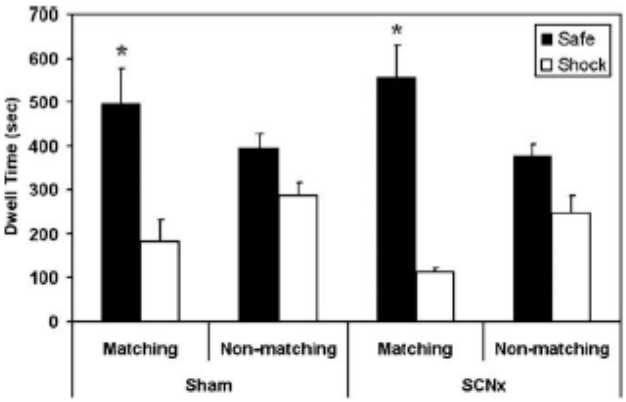


Figure 13

Mice were testested for conditioned place avoidance. Two rooms, connected by a tunnel, were different in three ways (smell, wall pattern and size). Aniamsl were conditioned by placing them in room A and room B in alterating order. In one room the animal was 'safe' and in the other room the animal received mild shock. After training the animals were reintroduced to both rooms, by opening a tunnel. If this introduction was at the same time of conditioning both SCN leasioned avoided the 'shock room'. If the times didn't match the borh groups showed no significant avoidance of certain places. This shows without SCN animals can still form time-place associations.

Also other clock gene impairments affect time place learning in similar ways *PAS2*$^{-/-}$ mice had impairments in cued conditional fear tasks. [Garcia JA *et al.*, 2000]. Also *Cry*$^{-/-}$ mice showed an inability to learn time place associations (see figure 12) [Van der Zee EA *et al.*, 2008].

Forming time-place associations in the memory is independent from the SCN. This was shown by Cain *et al.* in a time-place avoidance test (figure 13). Both control and SCN lesioned mice showed conditioned place avoidance if the time of testing was matched to the time of conditioning [Cain SW and Ralph MR, 2009].

Combined these studies show that memory formation, and specific time place learning, depends on circadian clock mechanisms that are located outside the SCN. Both the stimulus assessment in the forebrain, were NPAS2 is involved in circadian rhythmicity, and circadian rhythmicity in the hippocampus might be essential for the formation of time based memories.

The clock in the testes?

Although clock genes are mainly involved in circadian timing some contradictory results were found in the mammalian testes. The testes has two major functions; spermatogenesis and androgen production, witch in turn are tightly linked. In 1998 Zylka *et al.* looked at multiple tissues in relation to the expression of *Period* genes. They found circadian rhythms in the expression of *Per1, Per2* and *Per3* with different phases in skeletal muscle, liver, lung and testes. This was, to our knowledge, the first article showing a circadian rhythm in clock gene expression in the testes. Figure 8 shows the data, derived from mPer detection in western blot by radioactive mPer probes, as relative expression [Zylka MJ *et al.*, 1998].

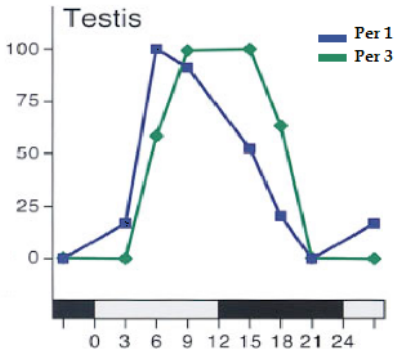


Figure 8

Five years later Morse found the circadian clock genes *Per1, BMAL1* and clock are all expressed in testes extracts, but in opposite of the findings of Zylka, they showed a constant level of expression and no circadian rhythm. Also they found *Clock* and *Per1* transcripts were present in different cell types (spermatids contained *Per1,* spermatocytes expressed *Clock).* Normally when *Clock* and *BMAL*1 act as a dimer activating Per transcription, here Per was present without Clock mediated transcription. This was also shown in *Clock*$^{-/-}$ mice, which still had high, and similar to

wild type, levels of *Per* in spermatids [Morse D *et al.*, 2003].

The expression of *Per1*, in stages VII and VIII in mice, coincides with the translation of an transcription factor known as CREM [Delmas V *et al.*, 1993]. This transcription factor can bind to CRE- elements, which are also present in the promoter regions of the *Period* genes. Transcription of *period* genes can also be influenced by cAMP. This suggests clock genes not necessarily need other clock genes to be expressed, and have functions other than reflecting the phase of a circadian rhythm, to time intracellular processes.

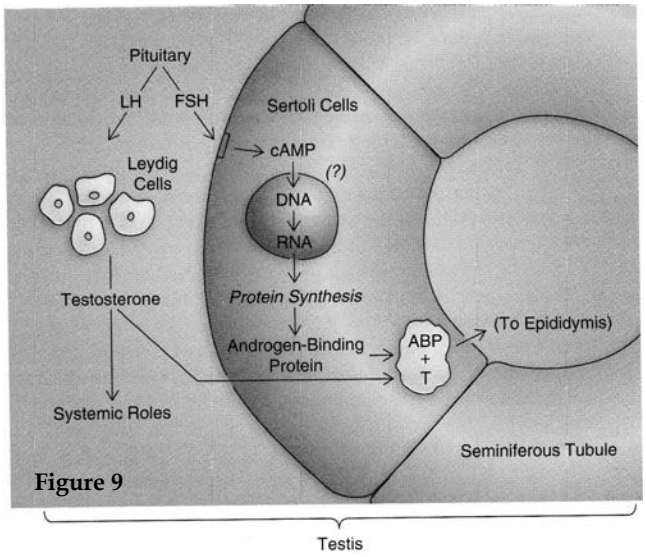


Figure 9

Whereas *Clock* is only present in the early cells of spermatogenesis, before meiotic division, *Per* is present after meiosis in stages VII-IX. The exact function of either *clock* or *Per* in spermatogenesis is currently unknown. Their 'normal' function in producing oscillations in an individual cell by feedback loops seems absent in spermatogenesis. [Morse D *et al.*, 2003].

Alvarez found that homozygous *BMAL*$^{-/-}$ mice, both male and female, are infertile, and showed serious impairments in steroid genesis. Testosterone producing cells in the testes, called Leydig Cells, show a circadian rhythm in BMAL expression. Also a direct effect of BMAL on an key enzyme in testosterone production (StAR) is shown in vitro; BMAL enhanced *StAR* transcription. Male *BMAL*$^{-/-}$ mice had high plasma LH levels, but almost no testosterone production. [Alvarez JD *et al.*, 2008]. Since testosterone is essential for primary spermatid migration towards the lumen of the seminiferous tubule, the infertility of *BMAL*$^{-/-}$ mice can thus be explained.

They role of clock genes in the testis is poorly understood. Taken together the Sertolli cells, and the spermatocytes they nurture, are showing no direct circadian rhythms. Instead the core clock gene levels look more dependent on developmental stages. Because Sertolli cells, and spermatocytes largely outnumber the Leydig cells in testicular tissues, extracts from the whole testes show a arrhythmic clock gene expression profile as found by Morse [Morse D *et al.*, 2003].

It might be also possible differences in tissue preparation (loss of certain cell types by chemical threatments) tissue composition (cross section or Leydig cell dense encapsulating tissue) and analysis techniques and methods might account for the opposite findings of Zylka and Morse (circadian rhythm in *Per1 and 3* expression, versus non-rhythmic tissue). The very different findings are puzzeling indeed. Unfortunatly the part of Zylka research dedicated to testes analysis is marginal because the focus of his research was *period* expression in many different tissues.

When looking at the testosterone producing Leydig cells alone circadian rhythms in *BMAL* expression have been established, although this rhythm is undetectable in extracts of the whole testes [Alvarez JD *et al.*, 2003; Alvarez JD *et al.*, 2008]. About the expression patterns of other clock genes in the Leydig cells no current literature exists. When we want to expand our knowledge about clock gene function in the testis it is important to first dissociate between different cells types and second note that expression of clock genes might be spermatogenesis-stage specific. Also the circadian clock genes might perform different functions, and show different interactions, between tissues [Looby P and Loudon AS, 2005].

In conclusion

The SCN forms an important player in the complex regulation of circadian rhythms in physiology and behaviour. Although tissues outside the SCN express often the same clock genes as the SCN, recent work has shown that local (peripheral) gene expression rhythms might play more important roles as expected. The exact role(s) of clock genes in the retina, allthough perheps te most studied peripheral oscillator, is still not clear to see.

Food driven rhythms look to depend on another type of 'circadian' oscillator, because without the SCN, animals can retain behaviour rhythms by food regimes. Where this food, or stimulus, driven oscillator seats the mammalian body remains unclear. Possibly it comprises both the circadian rhythms in local tissues, sensitive to food or other stimuli, which interact with SCN signals.

NPAS2 and *CLOCK* might represent two opposites in stimulus and light driven rhythms, because they are functional simular, but mutant phenotypes show subtile difference in the succes of either light or food entrainment. For organ and tissue function clock genes play different, and often poorly understood roles. The presence of clock genes in the testes for example seem to be more related the developmental stage of the spermatocytes, than to any circadian rhythm. Certain clock genes might be essential in one tissue, but replacable in others.

Clock genes are more than a oscillating loop of transcription and inhibition factors of each other, and also affect activity or expression of other genes, like AANAT, involved in retinal function. Understanding circadian rhythmicity at tissue level might provide new viewpoints on many healthproblems. Ranging from sleep and metabolic disorders to the timing of drug treatments or surgery in specific tissues. In unraffeling the body wide web of clock gene interactions and their role and functions in local tissues, lies still an daunting task.

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Beoordelingsformulier

Bijlage IV Bachelorscriptie: beoordeling en evaluatie

Onderstaande tabel geeft een overzicht van de criteria waarop je voor de bachelorscriptie wordt beoordeeld. Deze criteria zijn onderdeel van het tentamenbewijs dat in het bijzijn van de student wordt ingevuld en besproken.

	onvoldoende	voldoende	ruim voldoende	goed	excellent
1. Inhoud					
• Methode van aanpak	□	□	□	X	□
• Afbakening onderwerp/probleemstelling	□	□	□	X	□
• Relevantie behandelde onderwerpen en deelvragen	□	□	□	X	□
• Analyse en interpretatie van gegevens/informatie	□	□	□	X	□
• Kritisch verwerken van informatie	□	□	□	□	X
• Aansluiting bij/toepassing van bestaande theorie	□	□	□	□	X
• Breedte en diepgang	□	□	□	□	X
• Originaliteit	□	□	X	□	□
Beoordeling inhoud:					
2. Verantwoording					
• Verantwoording aanpak / planning / design	□	□	□	X	□
• Consistentie en onderbouwing van argumentatie en conclusies	□	□	□	□	X
• Keuze en gebruik van referenties / bronnen	□	□	□	X	□
Beoordeling verantwoording:					
3. Vormgeving/schriftelijke presentatie					
• Structuur / verhaallijn / opbouw betoog	□	□	□	□	X
• Stijl en formulering (uitdrukkingsvaardigheid)	□	□	□	□	X
• Spelling en grammatica	□	□	□	X	□
• Illustratie (gebruik grafieken, figuren, e.d.)	□	□	□	□	X
• Bronvermelding / referenties	□	□	□	□	X
• Omvang	□	□	□	□	X
Beoordeling vormgeving/presentatie:					

Sterke punten:
- Goed begrip materiaal
- Verliest zich niet in details - houd hoofdlijn vast.

Verdient bij volgende scriptie extra aandacht:
- Herlezen! Laat kleine teksten fouten eruit
- Figuur tekst.
- Eigen beredenering beargumenteren.

Opmerking Masterparcours:
- Cursus structuur waarbij research onderwerp parallel aan scriptie loopt heeft consequenties voor de kwaliteit van de scriptie.

Eindcijfer: 8 1/2

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begeleider: Roelof A. Hut
1-juli-2009