Instituto Juan March
de Estudios e Investigaciones

96 CENTRO DE REUNIONES INTERNACIONALES SOBRE BIOLOGÍA

Co-sponsored by

EMBO EUROPEAN MOLECULAR BIOLOGY ORGANIZATION

Workshop on

Molecular Clocks

Organized by

P. Sassone-Corsi and J. R. Naranjo

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Introduction

J. R. Naranjo and P. Sassone-Corsi

Circadian and seasonal rhythms are central to most biological systems, from daily oscillations in plant photosynthesis to hormone secretion and annual breeding cycles in mammals. All animals have an endogenous clock or *pacemaker*, which, independently from the day-night cycle, generates circadian rhythms in physiology and behaviour. For years researchers thought that these regulatory systems required an intact tissue organization and relied upon intercellular communications. Today we know that each cell composing the *pacemaker* has endogenous oscillatory properties and contains an autonomous clock.

During the past 10 years much attention has been given to the search for genes responsible for the clock function. Clock genes have been cloned in *Drosophila*, *Neurospora*, zebrafish and mammals. An emerging common feature is that most clock genes encode proteins with the structural characteristics of transcription factors, although there are notable exceptions. These transcription factors are characterized by the presence of PAS domains, a structural motif involved in protein-protein interactions. PAS domains take the name from the *Drosophila* PER gene, the mammalian Arnt (a dimerization partner of the dioxin receptor) and Sim, the product of the fly *single-minded* gene. In addition, a second general feature is that clock molecules operate within regulatory networks where autoregulatory feedback loops play a central role. The example of *Drosophila* PER and TIM - factors encoded by the *period* and *timeless* genes, respectively - represents a paradigm in the field.

The identification of molecular clock components has provided powerful tools to address fundamental biological questions such as: which cells contain clocks? When and how the clock starts ticking? How is it able to anticipate the light-dark cycle, and how is light able to directly influence clock function? These questions, and others, constituted the centre of debate during the recent "Molecular Clocks" meeting organized by the Juan March Foundation in Madrid, Spain. As a result, a base for consensus has been achieved for some of the major topics in clock function during these days of discussions. First, data from independent groups predicted the existence of extraretinal photoreceptors in mammals which would mediate entrainment of the clock to light-dark cycles, for instance reproductive responses to photoperiods. In this context, the identification of CRY1 and CRY2 in the mouse, which are homologs of

plant blue light-receptors (cryptochromes) and photolyases, open a new avenue of understanding since they are expressed in tissues that are not commonly though to be light-sensitive. Second, clock genes have a generalized expression in various tissues, and analysis of their subcellular location and protein-protein interaction has established a greater complexity in the regulation and combinatorial functions of these factors in mammals. Third, oscillators are present in peripheral tissues and oscillation is kept in *ex vivo* organ cultures of these tissues. Furthermore, independent pacemakers can also be revealed in single cells where their oscillations can be influenced in different ways by agents acting on various intracellular signaling pathways.

Our understanding of the functioning of the circadian clock is progressing by leaps and bounds and the future will undoubtedly hold many surprises. Crucial questions are: what is the biological role of peripheral clocks? How does light entrain the clock? How do the molecular components of the clock work together and how are they modulated by intracellular pathways?.

J.R. Naranjo and P. Sassone-Corsi

Session 1: The light signal

Chair: Martin Raff

Non-Visual Responses to Light: More Surprises

Michael Menaker

It is now commonly recognized that all vertebrates–with the single but important exception of mammals–have multiple photoreceptors outside of the retina. These extraretinal photoreceptors have been shown to mediate entrainment of circadian rhythms and reproductive responses to photoperiod as well as many other responses to light that do not require image formation. Although these responses have been studied for almost 100 years, the photoreceptive cells responsible for them have not been positively identified.

Early in their evolution, mammals passed through a "nocturnal bottleneck" which may have caused a reorganization of their entire circadian axis, in particular its photoreceptors. As far as we know, all modern mammals completely lack extraretinal photoreceptors; all responses to light are mediated by the retina, although not necessarily by the rods or cones. The mammalian pineal contains much of the machinery normally associated with phototransduction but apparently does not respond to light, although there have been sporadic reports of light responsiveness by neonatal rat pineals. I will discuss unpublished experiments in which we (Tosini and Menaker) have shown that robust light responsiveness can be induced in neonatal rat pineals by culturing them under appropriate conditions.

While the mammalian pineal is not normally photosensitive, it does participate directly in the reproductive response of seasonally breeding mammals to photoperiod. The photoperiodic signal, perceived by the retina, is processed by circadian oscillators in the suprachiasmatic nuclei (SCN), which in some unknown way determine its duration; the SCN then regulates pineal melatonin synthesis to reflect the prevailing day length, and the melatonin signal regulates the reproductive response. At least, this is the prevailing dogma. This model implies that the retinal photoreceptors which mediate circadian photoreception also mediate the reproductive response, since this response relies on circadian output from the SCN. I will report recent unpublished experiments in which we (Menaker, Ihara and Flari) have treated albino hamsters with bright constant light which damages their retinas. The results demonstrate that the circadian photoreceptors, by themselves, are not sufficient to support the reproductive response to light, since animals with retinas damaged by bright constant light entrain to light:dark cycles, but respond reproductively as if they were in constant darkness. This suggests strongly that the prevailing dogma is oversimplified.

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Control of Pineal Gland Molecular Rhythms

by
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An abundance of evidence indicates that the pincal gland is the major interfacing organ that enables light to regulate a varicty of biological rhythms. Light reaches the pineal gland through a circuitous route involving a retinohypothalmic pathway with neural information then proceeding down the intermedial lateral cell column of the spinal cord out to the superior cervical ganglion from which post-ganglionic fibers ascend to the pineal gland. The principal means whereby the pineal gland influences biological rhythms in other tissues involves its release of the hormone melatonin. Rhythms in melatonin formation are the most dramatic of all biological rhythms with hundred fold variations between nighttime peaks and daytime troughs.

Melatonin is formed from the neurotransmitter seroronin by N-acetylation using the enzyme serotonin N-acetyltransferase /NAT) followed by methylation of the 5-hydroxy group by hydroxyinodole-o-methyltransferase (HIOMT). NAT is the rate-limiting enzyme in the process with its activity peaking in the nighttime, while only negligible rhythms occur in HIOMT activity. A breakthrough in understanding regulation of these rhythms was the cloning of NAT (1,2). This made it possible to look for promoters that might provide pineal-specific and nighttime specific activity. To identify pineal-specific regulatory elements would require identication of a variety of pineal-specific proteins, especially those that undergo diurnal rhythms.

An approach utilized in our laboratory to identify nighttime specific pineal gene expression involved subtractive hybridization between day and night pineal gland RNA (1). Besides identifying NAT, we found at least two other night-specific genes. One is a novel pineal night-specific ATPase oncoded by the Wilson Disease (WD) gene (3). The WD gene is a copper transporter. PINA is an alternatively spliced form of WD, which lacks the copper sensor region. PINA rhythms in the pineal gland are as dramatic as those of NAT. Nocturnal pineal expression of PINA is controlled by the hypothalamic clock mediated via adrenergic innervation of the pineal gland. PINA does posses copper transport activity.

To identify regulatory elements for pineal-specific gene expression we isolated sequences upstream of the rat PINA gene and discovered a cis-acting element that is recognized by a novel PINA/retina-specific nuclear factor (4). This pineal regulatory element (PIRE) has a consensus sequence which is present in six copies of the 5' regulatory region of the PINA gene, at least three copies in the rat NAT promoter and at least one copy in the putative HIOMT promoter. PIRE interacts selectively with the cone-rod homeobox (CRX) which is specific for the retina and the pineal gland. Thus, CRX appears to play a crucial role in regulating pineal gene expression through interactions with PIRE.

Nighttime specificity of pineal gland expression depends on the nighttime augmentation of firing of the sympathetic innervation of the pineal gland. The resultant release of norepinephrine stimulates beta-adrenergic receptors to elevate cAMP levels in the pineal gland. cAMP activates cAMP-dependent protein kinase, which phosphorylates the CRE binding proteing (CREB) which in turn binds to CRE sites to activate transcription. Other regulators of CRE include cAMP response element modulators (CREM). The promoter region of NAT contains CRE elements, which bind various forms of CREM as well as CREB and thus presumably regulate nighttime specific expression.

Recently we identified a dramatic nighttime expression in the pineal gland of the tumor suppressor Patched-1 (PTC-1) a product of the mammalian homolog of the Drosophila segment polarity gene patched (5). Patched is a receptor for Hedgehog, which is crucial for embryonic development. Although little is known about the signal transduction pathways activating PTC-1, its transcriptional augmentation has always been associated with elevated Hedgehog activity. We demonstrated a dramatic diurnal rhythm in PTC-1 expression in the pineal gland with the peak at midnight. Like other rhythms, this one is regulated by adrenergic innervation of the pineal. Strinkingly, PTC-1 transcription is independent of Hedgehog signaling with no Hedgehog detectable in the pineal gland. Instead, it is induced by agents that activate the cAMP signal transduction pathway.

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Russell G. Foster: Mammalian Photoentrainment: Fact and Fantasy

Light is an influential regulator of physiology and behaviour in mammals. An important aspect of this regulation is the ability of the light environment to provide time of day information. This information is used to regulate temporal aspects of physiology and behaviour including entrainment of circadian clocks. Despite the importance of these processes, relatively little definitive information regarding the mechanisms of circadian photosensitivity are available. In particular, the photoreceptors which mediate these responses remain undefined.

Over the past few years there has been growing support for the hypothesis that the photoreceptors mediating circadian responses differ from the classical photoreceptors of the visual system. A large part of the justification for this hypothesis has been the observation that, in mice, degeneration of rod and cone photoreceptors, and loss of visual responses, is not necessarily associated with a reduction in the sensitivity of circadian responses to light. Most recently, we have demonstrated that mice completely lacking both rod and cone photoreceptors are capable of exhibiting photoentrainment [1] and suppressing pineal melatonin by light [2].

The exciting conclusion that mammals may contain a previously uncharacterised photoreceptor has led to considerable speculation regarding its nature. On the basis of recent tract tracing studies, retinal ganglion and/or amacrine cells surfaced as strong candidates [4]. In addition, a variety of candidate non-rod, non-cone photopigments have been suggested, the most recent being the mammalian cryptochromes (CRY1 and 2), vitamin B2-based putative photopigments [7]. Mouse *cry1* and *2* genes are expressed within the inner retina and retinal ganglion cells (amongst many other sites in the body) [3]. Other candidates include two non-rod, non-cone photopigments of the classical opsin:vitamin-A family that have recently been identified in non-mammalian vertebrates [5, 6]. Both genes are expressed in cells of the retinal inner nuclear layer outside of the classical photoreceptors. Presently, there is limited direct

evidence linking any of these putative novel photopigments with circadian photoreception. We anticipate that studies on rodless, coneless mice will prove successful in addressing this deficit. For example, our demonstration that these mice are highly sensitive to monochromatic 509 nm light already excludes those photopigments whose absorbance spectrum does not encompass this wavelength.

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Mammalian blue-light photoreceptor homologs CRY1 and CRY2 are essential for maintenance of circadian rhythms

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Many biochemical, physiological and behavioural processes exhibit circadian rhythms, generated by an internal time-keeping mechanism referred to as the biological clock. According to rapidly developing models the core oscillator driving this clock is composed of an autoregulatory transcription-(post)translation based feedback loop involving a set of 'clock' genes. Molecular clocks do not oscillate with an exact 24-hour rhythmicity but are entrained to solar day/night rhythms by light. Recently, mammalian CRY1 and CRY2, members of the family of plant blue light-receptors (*cry*ptochromes) and photolyases, have been put forward as candidate light-receptors for photoentrainment of the biological clock. We recently demonstrated that mice lacking the CRY1 or CRY2 protein exhibit accelerated and delayed free-running periodicity of locomotor activity, respectively. Strikingly, in the absence of both proteins, an instantaneous and complete loss of free-running rhythmicity is observed. In the presence of only one intact *Cry1* allele, the clock keeps running for a limited number of cycles in DD, indicating a direct involvement in the clock system and a gene-dosage effect. Our findings suggest that in addition to a possible photoreceptor and antagonistic clock-resetting function, Cry proteins are essential components of the clock itself.

Session 2: Functional conservation of the clock

Chair: Michael Menaker

Molecular Genetics of Circadian rhythms in *Drosophila*

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Genetic and Molecular analyses of the *Drosophila* circadian system identified the *period* and *timeless* proteins (PER and TIM) as clock molecules that contribute to circadian pacemaker function.. Both genes show robust circadian rhythms of transcription, mRNA and protein expression. Furthermore, the two proteins interact to form a heterodimeric complex, and TIM levels respond to light, thereby tying the circadian pacemaker to photic stimuli. More recent work has identified four new *Drosophila* clock genes: *Clock*, *cycle*, *doubletime*, and *cry*. *Clk* and *cycle* are bHLH-PAS transcription factors. These heterodimeric partners drive PER and TIM expression, in the mammalian as well as the *Drosophila* system. *doubletime* is a kinase implicated in PER phosphorylation, and *cry* is a photoreceptor that connects the molecular clock components to the major environmental entraining stimulus, light. There is evidence that *cry* contributes to circadian light perception in mammals and plants as well as *Drosophila* and is thus the first clock molecule to cross the plant-animal boundary. Despite this substantial recent progress, there are a large number of questions that remain unanswered. These include the feedback effect of PER and TIM on CLK and CYC activity, the post-transcriptional regulation of clock proteins, identification of the cells that drive locomotor activity rhythms, the multiple inputs that entrain these rhythms, and the outputs required for their detection. In the latter case, some of these operate far downstream of the transcriptional events that have been a major focus of the field. New features of these issues, especially input and output, will be discussed. Finally, some evolutionary issues will be considered, in an attempt to provide a framework for considering together different circadian systems.

Novel components of *Drosophila's* circadian system identified by tracking rhythmic gene expression in live flies

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Circadian systems allow organisms to adapt their lives to the daily occurring environmental changes of light and temperature. Three different components, which interact with and feed back on each other, are common to all known circadian systems: (1) the "endogenous clock", generating circadian oscillations, (2) entrainment pathways, through which environmental stimuli, such as light and temperature (=Zeitgebers), reset the clock, and (3) output pathways that signal the time information from the clock to effector systems, those that display overt features of biological rhythmicity (e.g. locomotor activity, leaf movement).

Despite recent progress in identifying important new players in the molecular clockworks of *Drosophila* (*Clock, cycle,* and *doubletime*) and their interactions with the classic clock components *period* and *timeless* (recently reviewed by Dunlap, 1999), there are still many questions to be answered and missing links to be filled in order to understand and assemble the flies endogenous clock, let alone the whole circadian system. For example we have only begun to understand how light is transmitted to the clockworks (see below), a process ultimately resulting in TIM protein disappearance and clock resetting (Dunlap, 1999). Moreover very little is known about clock output processes. Looking at the situation in mammals and *Neurospora*, it can be speculated that rhythmic expression of downstream genes, controlled by the same feedback loop running the central clock (Jin et al., 1999), plays an important role at this level of the circadian system (see below).

In an attempt to identify new molecules involved in the circadian system we performed novel mutagenesis screens based on monitoring rhythmic gene expression in live flies, using the firefly luciferase reporter gene (Brandes et al., 1996). One of these screens was aimed to identify mutants that act upstream of *per* (input pathway), or at the level of the clock itself. To do this we chemically mutagenized flies expressing a *period-luciferase* fusion gene, which normally gives rise to robust bioluminescence rhythms due to *per's* rhythmic expression. We screened for mutants that somehow alter or abolish these bioluminescence rhythms; therefore we were looking for defects in clock-gene expression, instead of applying the usual screening strategy that involves last-stage features of clock output (behavioral or eclosion defects). Among ca. 5000 lines tested, we identified a mutation that results in arrhythmic expression of the *period* gene; the mutation turned out to be in the *Drosophila cryptochrome* gene (Stanewsky et al., 1998; Emery et al., 1998). Analysis of the cry^b mutant revealed that the *cry* gene, encoding a putative blue-light photoreptor, is important for light entrainment. It also seems to effect clock function, since *per* and *tim* expression are arrhythmic in peripheral clock cells. However cry^b does not effect rhythmic locomotor behavior under constant conditions, and as expected from this result, PER and TIM proteins still oscillate in a subset of *Drosophila's* brain pacemaker cells, the so-called Lateral Neurons. Since PER and TIM expression in these mutant cells can still be entrained by light/dark cycles, they somehow must receive light signals. One possibility would be that light received by opsins in the external photoreceptors is transmitted to the Lateral Neurons. Indeed, we found that flies mutant for *norpA* (defective in the rhodopsin phototransduction cascade) and cry^b exhibit more dramatic entrainment defects compared to either mutant alone (pointing to an involvement of opsin photoreceptors in fly entrainment). These double mutants have now been tested for TIM cycling in the Lateral Neurons (so far only the behavior of *norpA;* cry^b flies was studied: Stanewsky et al., 1998); they still showed robust oscillations of this protein, suggesting the existence of yet unidentified photoreceptive structures and/or molecules.

From studies in mammals, *Neurospora* and *Arabidopsis* it is known that rhythmic expression of so called *clock regulated genes* (crg's), is a way to establish clock output functions. In flies, two putative crg's (*dreg5* and *crg1*) were isolated using methods based on isolation of differentially expressed RNA's. Their rhythmic expression was shown to be dependent on *per* and *tim* gene function, indicating that they are indeed crg's (van Gelder and Krasnow, 1996; Rouyer et al., 1997). Since no mutants are available for *dreg5* and *crg1* their functions remain unknown, and it is not clear on what level of the circadian system they might act. That is, *dreg5* and *crg1* could be clock genes instead of output factors. In order to study how clock output is organized in *Drosophila* we sought a way that would allow us to isolate rhythmically expressed genes and at the same time to determine their function by mutational analysis. We developed a mutagenesis screen based on mobilizing a *luciferase* encoding transposable element in the fly's genome. The element is a modified version of a classical *lacZ* P-element enhancer-trap vector, which was used in the past to identify genes that function during development (e.g., Bier et al., 1989). In these screens, many strains have been identified in which the reporter-containing transposon inserted at a genetic locus (usually in a region flanking the coding one) and β-Gal expression was under the control of the gene's regulatory sequences. By analogy we expected our *luciferase* P-element to insert next to transcribed sequences, and automated monitoring of the temporal bioluminescence expression should permit us to identify rhythmically expressed genes. Since the features of the P-element allow the isolation of flanking genomic sequences and their deletion (by imprecise excisions of the transposon, thereby creating potential deletions in the gene of interest), it is possible to clone the rhythmically expressed genes and to determine their potential function in the circadian system.

By identifying new molecular components and their biological function, both approaches combined should lead to a more complete understanding of *Drosophila*'s circadian system.

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Molecular Genetics of Circadian Rhythms in Mammals: Mouse *Clock* Gene as a Master Regulator of Circadian Pacemaker

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In order to search for mammalian clock genes, we initiated an ENU mutagenesis screen for circadian phenotype. In this screen, we isolated the first circadian mutation in mice that is named *Clock*. The *Clock* mutation is semidominant and causes lengthening of the circadian period by 1 hour in heterezygotes and by 4 hours in homozygotes. Importantly *Clock* homozygots lose persistent circadian rhythms after longer exposure to constant darkness conditions (Vitaterna et al., 1994). Using a positional cloning strategy in combination with the functional rescue of the mutation by transgenic expression in mice of large genomic BAC clones, we identified the gene encoding the *Clock* mutation (King et al., 1997; Antoch et al., 1997). The gene is represented by a single transcription unit that spans ~ 100,000 kbp and contains 24 exons. This transcription unit encodes a novel member of the basic helix-loop-helix (bHLH)-PAS domain family of transcription factors. In the ENU-induced *Clock* mutant allele, we identified a single A to T nucleotide transversion in a donor splice site that results in the skipping of exon 19. This in turn leads to deletion of 51 amino acids in the C-terminal region of CLOCK protein in the putative transactivation domain. It has been also shown that CLOCK protein dimerizes with another bHLH-PAS protein known as BMAL1 (Gekakis et al., 1997, Hoganesch et al., 1997). The CLOCK-BMAL1 heterodimer binds to and transactivates through an E-box motif (-CACGTG-) found in the *period* gene promoters of both Drosophila and mice. Recent cloning of mammalian orthologs of the Drosophila *timeless* gene and demonstration that mammalian TIM and PER negatively regulate transcription via the m*Per1* promoter, "closed" the mammalian circadian loop (Sangoram et al., 1998).

To date, *Clock* represents the only member of bHLH-PAS domain transcription factor family which has been demonstrated to affect mammalian circadian clock function. To study in a more detailed way how *Clock* regulates the circadian system, we examined both overt (behavioral) and molecular (gene expression in the SCN) rhythmic responses to light pulses of wild-type and *Clock* mutant mice by generating the phase response curves (PRCs) to saturating light pulses. We demonstrate that higher amplitude PRC was apparent in *Clock* heterozygous mutants. This was accompanied with the lower levels of m*Per1* gene expression in the SCN of *Clock* mutant mice. At the same time, acute m*Per1* induction by light remains unaffected in *Clock* heterozygotes (however, m*Per1* response is lower in *Clock* homozygotes). M*Per1* message has been shown to oscillate in the SCN in constant darkness and to be rapidly induced by light (Albrecht et al., 1997; Shigeyoshi et al., 1997; Zylka et al, 1998) at phases at which light resets thus representing the photically responsive element of the mammalian circadian oscillator. Our findings on PRC differences in wild-type and *Clock* mutant mice are consistent with the hypothesis that the *Clock* mutation alters the PRC by reducing pacemaker amplitude. It represents the first *in vivo* demonstration that *Clock* positively regulates m*Per1* expression. Interestingly, two other members of *Per* genes family, m*Per2* and m*Per3*, appear to have a comparable to m*Per1* reduction in amplitude of expression in the SCN in *Clock* mutant mice. And finally, in *Clock* mutants all three *Per* genes have reduced levels of expression in all peripheral tissues that have been shown to exhibit circadian oscillation (Zylka et al., 1998). All these findings suggest a role for the *Clock* gene as a master regulator of circadian function and provide the possible mechanisms of this regulation.

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Photoperiodically induced changes in gene expression in Sinapis alba and Arabidopsis thaliana

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Sinapis alba can be induced to flower by a single 22 hour long day. During this long day, an endogenous signal called the floral stimulus should be produced in the leaf where the change in photoperiod is perceived, in order to stimulate flowering at the apex. It has been shown that in short day about 10% of the leaf mRNAs display a diurnal rhythmicity in their expression level. During the inductive long day, some of these mRNAs are differentially regulated and some others that do not show a rhythm in short day display an increased or a decreased expression level.
A Sinapis leaf cDNA library has been differentially screened with a short day and a long day probe. We isolated a series of 130 differentially regulated Sinapis cDNAs and concentrated on those showing the strongest and earliest changes. These mRNAs show an increased expression during the additional hours of light of the long day. In addition, some of these mRNAs also show an increased expression when flowering is induced by a displaced short day, a treatment allowing flower induction without additional light.
We are following a reverse genetic approach to study the putative involvement of these genes in the flowering process. We have cloned and sequenced Arabidopsis homologues of some of these cDNAs and studied their expression in the Arabidopsis one long day inductive system (Corbesier et al., 1996). Some of the isolated Arabidopsis genes also present an increased expression during the inductive long day.
Transgenic plants overexpressing sense or antisense transcripts of an unknown, photoperiodically regulated gene, SAH9, have been constructed. The flowering behavior of these plants will be analyzed. The same procedure will be used to analyze the other photoperiodically induced genes.
In order to determine which sequence motives are necessary for photoperiodic induction, a promoter deletion study has been undertaken with the SAH9 promoter as well as with another photoperiodically induced gene promoter. Trans acting factors are searched using EMSA in order to isolate transcription factors that regulate the process.
In another approach to better characterize the cloned genes, mutants containing an En transposon insertion in the gene of interest have been searched and some positive candidates isolated. One of these candidates seems to have a late flowering phenotype.
Finally we have transformed promoter-conditional lethal genes fusions in Arabidopsis. After mutagenesis, these plants will be screened for mutants in the photoperiodic induction process.

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Session 3: Clocks are everywhere

Chair: Solomon H. Snyder

MOLECULAR ANALYSIS OF CLOCK GENES

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Mammalian homologs of Drosophila *per* and *tim* have been recently cloned and characterized. A family of three mouse *(m)Per* genes has been most extensively evaluated, with each found to exhibit circadian oscillations in RNA levels in the suprachiasmatic nuclei (SCN)(Sun et al., 1997; Tei et al., 1997; Albrecht et al., 1997; Shearman et al., 1997; Zylka et al., 1998a; Takumi et al., 1998). Mammalian CLOCK-BMAL1 heterodimers bind E box elements in the 5' flanking region of the mPer1 gene to activate transcription (Gekakis et al., 1998). A mouse *(m)Tim* homolog has also been recently cloned and characterized (e.g., Sangoram et al., 1998; Zylka et al., 1998b). In contrast to the three *mPer* genes, however, *mTim* RNA and protein levels are low and non-rhythmic in the SCN. In vitro transcriptional studies have nonetheless shown that each of the three mPER proteins and mTIM can negatively regulated CLOCK-BMAL1-mediated transcription (Sangoram et al., 1998; Jin et al., 1999). Homodimeric and heterodimeric interactions between the three mPER proteins have been described that may be important for their nuclear translocation and subsequent participation in negative feedback (Zylka et al., 1998b).

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ZEBRAFISH *Clock* GENE EXPRESSION: INDEPENDENT PERIPHERAL PACEMAKERS.

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The cloning of the first circadian clock gene in a vertebrate, the mouse*clock* gene, was the result of a large scale mutant screen. This work confirms the power of the genetic screening approach to identify the molecular components of the circadian clock. A potential alternative vertebrate model system for genetic screening analysis is the zebrafish (*Danio rerio*). The zebrafish has become a powerful model system to study early vertebrate development. For these types of studies, the zebrafish has the advantage that its early development is extremely rapid (from fertilization to hatching from the egg in 3 days) and that the embryos are transparent so they can be easily observed. However, a variety of techniques such as transgenesis and genetic screening are now being developed rapidly for zebrafish, so making it a useful model to explore many other biological processes.

The zebrafish potentially provides two advantages for studying the circadian clock. Firstly, it constitutes an alternative genetic model system to the mouse to identify new clock molecules via mutant screening. Secondly, it represents an ideal tool to examine the origin and role of the clock during early, embryonic development. Originally, it was demonstrated that the eyes and the pineal gland of this fish contain an endogenous circadian clock capable of directing rhythmic synthesis of the hormone, melatonin, in organ culture. In addition, circadian rhythms in the locomotor activity of zebrafish adults and larvae have been described. However, little is known about clock function in the zebrafish at the molecular level.

As a first step to develop the zebrafish as a model to study the circadian clock, we chose to characterize the zebrafish homologue of the mouse *clock* gene. Low stringency screening of a zebrafish embryo cDNA library resulted in the cloning of a zebrafish homolog of the *clock* gene, that shows a high degree of similarity to the mouse counterpart. RNase protection analysis revealed that the *clock* transcript oscillates with a pronounced circadian rhythm in the eye and pineal gland of the adult fish. This is in sharp contrast to the situation in the mouse, where *clock* expression shows no day-night changes. Further examination revealed that *clock* mRNA also oscillates with a circadian rhythm in many tissues within the adult fish. This observation raises the possibility that each tissue may contain its own circadian oscillator, or that these peripheral oscillations are driven from a central "master" clock. To answer this question we placed several tissues into culture in constant darkness, and, at a number of phases, RNA was extracted and the level of *clock* transcript was assayed. The oscillation of *clock* observed in vivo was also clearly apparent in vitro, in

the absence of any timing cues. We can conclude that the heart and kidney of the zebrafish do, in fact, contain an endogenous circadian oscillator.

Now, in posession of an oscillating clock component in the zebrafish, we envisage many possible lines of study. Defining the mechanisms driving rhythmic clock expression as well as the partners which interact with the clock protein may potentially lead to the identification of new clock components or regulatory systems. Furthermore, exploiting the *clock* promoter to drive expression of a fluorescent reporter gene in the context of a stable transgenic fish, could provide a valuable tool for a full-scale mutant screen.

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AN INTRINSIC TIMER AND EXTRINSIC SIGNALS CONTROL THE TIMING OLIGODENDROCYTE DIFFERENTIATION

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In most vertebrate cell lineages precursors cells divide a limited number of times before they stop and differentiate. In no case is it clear why the cells stop dividing when they do. We have been studying the timing mechanism in the oligodendrocyte lineage in the rodent optic nerve.

The oligodendrocytes develop from dividing precursor cells that migrate into the optic nerve early in development. The precursor cells are stimulated to divide mainly by PDGF, but even when they are cultured in saturating amounts of PDGF, the precursor cells isolated from a postnatal day 7 nerve divide no more than 8 times before they stop and differentiate(1). The timing of this cell-cycle arrest and differentiation depends on an intrinsic timer (1, 2), which consists of at least two components— a counting component that measures elapsed time and an effector component that stops the cell cycle and initiates differentiation when time is reached(1). I shall present evidence that the effector mechanism is regulated by thyroid hormone(1), that an increase in both the cyclin-dependent kinase inhibitor p27 and a thyroid receptor protein is part of the timer (3, 4, 5, 6), and that the increase in p27 is controlled post-transcriptionally while that of the thyroid receptor is controlled transcriptionally.

It seems very likely that similar mechanisms operate in many other cell lineages.

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Session 4: Clock molecular networks

Chair: Paolo Sassone-Corsi

Transcriptional Regulation inVertebrate Circadian Clocks

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The mouse *Clock* gene encodes a bHLH-PAS protein that regulates circadian rhythms and is related to transcription factors that act as heterodimers. A heterodimeric partner of CLOCK, BMAL1, was isolated in a two-hybrid screen. CLOCK-BMAL1 heterodimers bound to and activated transcription from E-box elements adjacent to the mouse *Per1* gene and from an identical E-box known to be important for *per* gene expression in *Drosophila*. Mutant CLOCK from the dominant-negative *Clock* allele and BMAL1 formed heterodimers that bound DNA but failed to activate transcription. Thus CLOCK-BMAL1 heterodimers appear to drive the positive component of *per* transcriptional oscillations, which are thought to underlie circadian rhythmicity.

In transfection studies, expression of the mouse mPER1 or mPER2 proteins was found to inhibit *mPer1* gene activation by CLOCK-BMAL1, providing direct evidence for PER protein negative feedback on mammalian *Per* gene transcription. Protein interaction experiments and further analysis of PER inhibition of CLOCK-BMAL1 action suggest that PER binds to the CLOCK-BMAL1 heterodimer in a manner that prevents the transcription factor from binding to its E-box target site.

MOLECULAR SCREENS FOR NEW PIECES OF THE *Drosophila* CLOCK

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We are isolating novel clock-controlled genes in *Drosophila* that could either be part of the central clock mechanism or function in the output pathway from the circadian pacemaker. Using differential display of expressed genes, we isolated *vrille* (*vri*), a transcription factor previously identified with a role in development. Intriguingly, *vri* shows homology to DBP, a mammalian transcription factor that oscillates in the SCN. We show that *vri* is expressed in clock cells in both adult and developing flies and that *vri* RNA levels oscillate in synchrony with the *period* (*per*) and *timeless* (*tim*) genes in heads and bodies of wild type flies. *vri* RNA levels are constitutively low in *Clock* and *cycle* mutant flies, suggesting that *vri* is a direct target of the of the dCLK/CYC heterodimer. Indeed, we show that the *vri* promoter contains a single functional dCLK/CYC binding site. Therefore, the same transcriptional loop that maintains 24 hour cycling of the *per* and *tim* genes also regulates *vri* expression.

Flies with only one copy of the *vri* gene have a shortened period of behavioral rhythms, which is striking since mice lacking DBP also have shorter periods. This suggests that *vri* regulates the levels of a critical clock component(s). We show a genetic interaction between *vri* and *tim*, but not between *vri* and *per*. Consistent with this, we have found several potential VRI binding sites in the *tim* promoter. In mammals, DBP, TEF and HLF are part of a family of related proteins that oscillate and can form heterodimers with one another. Our difference screens have also recovered a gene, *Pdp1*, that encodes a *Drosophila* HLF homologue whose RNA oscillates in a clock-dependent manner in phase with *vri* . *Pdp1* expression was found to cycle robustly in pacemaker cells. We are currently testing for interactions between VRI and PDP1.

Our observations strengthen the similarities between the *Drosophila* and mammalian clocks, and show that molecular approaches can provide a viable alternative method for recognizing genes involved in clock function. The approach may be most useful when subtle phenotypes and/or the lethality of null mutants tend to compromise the effectiveness of traditional genetic screening.

Circadian transcription within the *Drosophila* feedback loop: Roles and Mechanisms

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The circadian clock controls a wide array of biochemical, physiological and behavioral phenomena in prokaryotic and eukaryotic organisms. At the center of this clock lies the timekeeping mechanism, or oscillator, which is inherently intracellular and requires rhythmic macromolecular synthesis. In *Drosophila*, several genes required for oscillator function have been identified including *period* (*per*), *timeless* (*tim*), *dClock* (*dClk*), *Cycle* (*Cyc*), *double-time* (*dbt*) and *cryptochrome* (*cry*). The abundance of transcripts encoded by *per*, *tim*, *dClk* and *cry* cycle in a circadian manner with *per* and *tim* transcripts peaking early in the dark phase and *dClk* and *cry* peaking early in the light phase. These mRNA rhythms are regulated via circadian feedback, where PER and TIM proteins repress their own mRNA levels and augment the levels of *dClk* mRNA.

Regulation of *per* mRNA cycling has been studied in some detail and occurs primarily at the transcriptional level. We have identified a 69bp circadian regulatory sequence (CRS) upstream of *per* that can drive essentially normal circadian, developmental and spatial expression. Activation of *per* transcription requires dCLK and CYC, which dimerize and bind to an E-box (CACGTG) within the CRS. A similar E-box is found upstream of *tim*, and is also required for dCLK-CYC dependent activation. These results indicate that *dClk* and/or *Cyc* define the spatial and developmental expression of *per* and *tim*. Repression of *per* and *tim* transcription is mediated by PER and TIM proteins, which directly interact with dCLK and CYC.

Since E-boxes of the sequence CACGTG are present every ~4kb, the specificity for dCLK-CYC binding to the E-boxes upstream of *per* and *tim* must include other sequences. To determine which other sequences in the CRS are important for transcriptional activation, a series of eight CRS mutants have been generated and used to make transgenes that drive *lacZ* or *per* cDNA expression. As expected, mutants within the E-box show little, if any, *lacZ* expression. Expression is also abolished in the mutant immediately downstream of the E-box, but all other mutants show rhythmic expression similar to that of the wild type CRS. Likewise, mutants within, and immediately downstream of the E-box are not able to rescue rhythms in per^{01} flies, while other mutants rescue with similar efficiency as the wild type CRS. Thus, *in vivo*, sequences downstream of the E-box also contribute to dCLK-CYC dependent activation.

Though the E-box is necessary for expression in the context of the CRS, it does not appear to be necessary for rhythmic expression in the context of the entire *per* gene. A 13.2 kb *per* genomic DNA fragment was previously found to efficiently rescue behavioral rhythms in per^{01} flies. Two independent mutants that remove portions of the E-box have been made within this 13.2 kb DNA fragment and used to transform per^{01} flies. Both mutants rescue with similar efficiency as the wild type 13.2 kb fragment, but show long period phenotypes.

This result suggests that other regulatory elements are present in or around *per*. We are currently defining the molecular phenotypes of these mutants to determine whether transgene mRNA cycles and is in the normal spatial pattern.

Previous *per* transgenes that lack the CRS or only contain transcribed sequences are capable of rescuing rhythms in per^{01} flies. In addition, when *per* transcribed sequences are included in *per-luc* transgenes, the phase of mRNA cycling is altered. These results suggest that regulatory elements are present within the *per* transcribed region. Since removal of *per* intron 1 has been shown to severely decrease expression levels, we tested whether this intron contains regulatory elements that drive expression in locomotor activity pacemaker cells (i.e. LNs). *per* intron 1 was used to drive *lacZ* or *per* cDNA transcription from a heterologous basal promoter. This intron mediates *lacZ* expression in LNs, DNs and glia within the central brain and optic lobe as well as several groups of brains cells that do not normally express *per*. When *per* expression is driven by this intron *PER* accumulates rhythmically in LN's and rescues long *per*iod behavioral rhythms in per^{01} flies. Thus, *per* intron 1 contains regulatory elements sufficient for behavioral rescue. These internal regulatory elements may contribute to the normal phase and amplitude of *per* mRNA cycling.

The circadian feedback loop is present in many tissues in *Drosophila*, but only the LNs are known to mediate a rhythmic output: locomotor activity. Experiments with *per*-luc reporter genes showed that the antenna contains an autonomous circadian oscillator. To determine if there is a corresponding rhythm in chemosensory function in this tissue, we measured electroantennagram (EAG) responses to odors at different times of day. Rhythms in olfactory responses are observed in wild type flies during light-dark cycles and constant darkness, with the peak response occurring during the middle of the night. These rhythms are abolished in per^{01} and tim^{01} mutants and in 4 days of constant light. These results demonstrate that responses to olfactory cues are controlled by the circadian clock. Since olfaction is essential for food acquisition, social interactions and predator avoidance in many animals, circadian regulation of olfactory systems could have important effects on behavior.

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The RNA-binding protein *At*GRP7 - external regulation and autoregulation within the negative feedback loop

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Previously, we have identified the clock-regulated RNA-binding protein *At*GRP7 in *Arabidopsis thaliana*. In transgenic *Arabidopsis* plants that constitutively overexpress the RNA-binding protein, oscillations of the endogenous *Atgrp7* transcript are severely depressed whereas overexpression of a mutated cDNA which does not give rise to a functional protein does not affect the oscillations of the endogenous *Atgrp7* transcript. Taken together, these data indicate that both *Atgrp7* transcript and *At*GRP7 protein are linked in a negative autoregulatory circuit.

We have started to dissect the molecular mechanism underlying the *At*GRP7 feedback loop. *Atgrp7* oscillations are generated at the transcriptional level, as 1.5 kb of the promoter can confer circadian rhythmicity upon a linked beta-glucuronidase (gus) reporter gene. Within the promoter, we have identified a minimal clock response element mediating a low amplitude oscillation with peak expression in the evening and an additional element augmenting the amplitude. Genetic crosses between the *Atgrp7*-gus line and the *At*GRP7 overexpressors indicate that the promoter by itself does not mediate the negative feedback of *At*GRP7 on the oscillations of its own transcript.

Therefore, in wild type plants the *At*GRP7 feedback loop seems to be controlled in the following way: *Atgrp7* transcript levels are elevated through rhythmic transcriptional activation by the endogenous clock during the day. When significant *At*GRP7 protein has accumulated after a lag phase, *Atgrp7* mRNA abundance is limited at least partly through the *At*GRP7 protein itself by a posttranscriptional mechanism. In support of this, bacterially-expressed *At*GRP7 fusion protein has been shown to bind to its own RNA.

To delineate the *At*GRP7 target site, we have generated transgenic plants carrying chimeric genes including various parts of the transcribed region. These lines are being crossed with the *At*GRP7 overexpressors and the influence of an enhanced *At*GRP7 level on reporter transcript levels will be investigated. In this way, the target site of *At*GRP7 will be determined.

Session 5: Multiple cellular clocks

Chair: Steven M. Reppert

Circadian Gene Expression in Animals and Cells

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DBP, TEF, and HLF are related transcription factors of the PAR basic leucine zipper (PAR bZip) protein family. All three of these proteins accumulate according to robust circadian rhythms in liver and other peripheral tissues. PAR bZip mRNAs also oscillate in the suprachiasmatic nucleus (SCN) of the hypothalamus, thought to contain the central circadian pacemaker, and genetic loss-of-function experiments in transgenic mice suggest that all three of these proteins contribute to the determination of circadian period length (tau). However, all PAR bZip protein mutant animals display rhythmic locomotor activity and robust circadian gene expression in peripheral cells. Therefore, PAR bZIP family members are players of circadian output pathways, rather than central clock components. The same appears to be true for the orphan nuclear receptor Rev-Erbα, another circadian transcription factor under study. We have established rat and mouse tissue culture systems that mimic the circadian expression of genes observed in intact animals. Thus, after treatment of immortalized fibroblasts with high concentrations of serum, the levels of the mRNAs encoding Rev-Erbα, DBP, TEF, Period 1 and Period 2, cycle for several consecutive days in a circadian fashion. Biochemical fractionation of serum factors revealed multiple activities that can induce circadian rhythms in tissue culture cells. Likewise, several chemicals known for inducing different signal transduction pathways are capable of triggering circadian gene expression in vitro. The various signal transduction pathways that may be involved in eliciting circadian gene expression in this tissue culture system will be discussed.

DREAM: A DIRECT EFECTOR OF CALCIUM OSCILLATIONS ON GENE EXPRESSION

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By functional screening of a human caudate cDNA library we have recently cloned the *DREAM* gene that encodes the first known transcriptional repressor directly regulated by calcium influx to the nucleus (Carrión et al. Nature 398:80-84, 1999). Under basal conditions DREAM binds to the sequence specific regulatory element DRE and represses transcription of target genes containing the DRE. Upon stimulation, the rise of nuclear calcium is sensed by four EF-hands present in the DREAM protein inducing a conformational change of DREAM that results in unbinding from the DRE sequence and de-repression of the target gene. Furthermore, activation of PKA by increased cytosolic levels of cAMP also leads to unbinding of DREAM from DRE sites through a mechanism that is not totally undertood at the present (Carrión et al., Mol. Cell. Biol. 18:6921-6929, 1998). Since changes in calcium and cAMP levels within the SCN and pineal gland have been proposed to influence rhythmic gene expression in those tissues (D´Souza and Dryer, Nature 382:165-167, 1996; Nikaido and Takahashi, Neuron 3:609-619, 1989) we explored the possibility that DREAM participates in the control of circadian-related changes in gene expression.

Preliminary results show that a functional DRE is present within the P2 promoter of the *CREM* gene controling the expression of ICER-CREM isoforms known to be expressed rhytmically in the pineal gland. ICER modulates the oscillatory level of the hormon melatonin (Foulkes et al. TINS 20:487-492). Analysis of promoter regions of several genes known to oscillate during the photoperiod, will be presented and their regulation by DREAM discussed.

Resetting the clock cycle.

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The recently developed models for the mammalian circadian oscillation are based around the *Drosophila* and *Neurospora* precedents of autoregulatory transcriptional feedback loops (Rosato et al. 1997, Dunlap 1999). The recent cloning and characterisation of mammalian homologues of the fly *period* (Tei et al. 1987, Sun et al. 1987) and *timeless* (Zylka et al. 1998, Sangoram et al. 1998) genes has added further weight to these models. To date, the models have been based solely on patterns of expression of RNA in the surpachiasmatic nuclei (SCN), the principal circadian oscillator in mammals. This presentation will describe the characterisation of novel antisera against putative mammalian clock proteins. It will then consider temporal patterns of expression and cellular localisation of these proteins in the SCN and elsewhere in brain and pituitary. Finally, it will consider how the expression of these proteins is regulated *in vivo* by two classes of stimuli known to reset the mammalian clock: nocturnal exposure to light and enforced locomotor activity.

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Mammalian Circadian Autoregulatory Loop: A *Timeless* Ortholog and *mPer1* Interact and Negatively Regulate CLOCK-BMAL1-Induced Transcription

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We report the cloning and mapping of *mTim* and *hTIM* which are mouse and human orthologs of the Drosophila *timeless* (*dtim*) gene. The mammalian *Tim* genes are expressed in a wide variety of tissues including the suprachiasmatic nucleus of the hypothalamus and the retina. Unlike *dtim*, we find no evidence that *mTim* mRNA exhibits circadian oscillations. Importantly, hTIM mimics the function of dTIM in three different contexts. First, hTIM interacts with the Drosophila PERIOD (dPER) protein as well as the mouse PER1 and PER2 proteins in vitro. Second, hTIM and dPER interact in Drosophila (S2) cells and hTIM promotes nuclear entry of dPER. Finally, expression of hTIM and mPER1 specifically inhibit CLOCK-BMAL1-induced transactivation of the *mPer1* promoter. Taken together, these results demonstrate that *mTim* and *hTIM* are mammalian orthologs of *timeless* and provide a framework for a basic circadian autoregulatory loop in mammals.

POSTERS

Developmental timers in the oligodendrocyte lineage

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During vertebrate development, multipotential precursor cells undergo a limited number of cell divisions before they exit the cell cycle and terminally differentiate. It is unclear what causes the cells to stop dividing and differentiate when they do, although the mechanisms involved are important as they influence the number of differentiated cells produced and the schedule of normal development. We have been studying these mechanisms in the oligodendrocyte cell lineage, which is responsible for myelination in the vertebrate central nervous system.

Previous work has revealed that an intrinsic timer operates within oligodendrocyte precursor cells (OPC) to limit their proliferation. The timer is modulated by extracellular signals, including the major mitogen for this lineage, platelet-derived growth factor (PDGF), and thyroid hormone (TH). Two molecules are known to play a role in the functioning of the timer: the levels of the cyclin-dependent kinase inhibitor (CKI) $p27^{Kip1}$ and TH receptor β1 rise in OPC as their proliferative potential decreases. Moreover, OPC derived from $p27^{-/-}$ mice divide one or two more times than wild-type cells.

We are currently investigating the question of whether the timer primarily limits the proliferation of OPC with differentiation following as a consequence, or *vice versa*. We are studying the effects of prematurely inhibiting cell-cycle progression by over-expressing p27. To this end we have developed a novel retroviral system that allows the over-expression of p27 (or any gene of interest) and green fluorescent protein, which allows us to select infected cells in culture and observe their behaviour in real time. We are also using this system to studying other genes that may be involved in timing differentiation: for instance, other CKIs that may co-operate with p27 in limiting proliferation and the PDGFα receptor, which is normally down-regulated when OPC stop dividing and differentiate.

CIRCADIAN GENE EXPRESSION IN CANCER AND IN YOUNG ANIMALS

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In mammals, the suprachiasmatic nucleus (SCN) of the brain hypothalamus gland is the central pacemaker tissue of the body. Presumably, a synchronizing signal originates in the SCN and is transmitted to peripheral tissues. To better understand the mechanism of this transmission, we have analyzed circadian gene expression in cancerous tissues. Subcutaneous mammary injection of CL26 murine colon carcinoma cells into nude mice produced rapidly-dividing tumor masses. Though such tumors are well-supplied with blood by the mammary artery, they are not enervated by the central nervous system. In these tumors, PER1 mRNA levels displayed robust circadian rhythmicity. This result implies that the central signal emanated by the SCN is likely to be humoral. Interestingly, circadian output genes such as DBP, TEF, and Rev-ERBα displayed severely dampened cycling in the tumors, although their mRNA levels cycled normally in adjacent tissues of the same animals.

To further characterize the interplay between the brain and peripheral circadian gene expression, we have analyzed newborn animals, which do not display circadian behavior. Again, the PER1 gene displayed circadian rhythmicity even in peripheral tissues, but the circadian output genes DBP, TEF, and Rev-ERBα did not.

Thus, in cases of atypical tissue morphology and development (cancer) and atypical circadian behavior (infants), circadian expression of a central clock gene continues to be observed, while clock output genes lose their rhythmicity. In the case of infants, it is attractive to imagine that this observation correlates with its arhythmic behavior, useful for constant feeding. Further characterization of these cases of circadian rhythmicity has the possibility to better define the mechanisms of central and peripheral clocks and the communication channels between them.

CIRCADIAN CHANGES OF PRION PROTEIN mRNA IN THE RAT SUPRACHIASMATIC NUCLEI AND OTHER FOREBRAIN AREAS

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Although the expression of the normal prion protein (PrP^c) in the host is critical to the development of transmissible spongiform encephalopathies[1], the physiological role of this protein and the processes regulating its expression remain obscure. The possible involvement of PrP^c in circadian processes has been indicated by the discovery that mice devoid of PrP^c show altered circadian activity rhythms[2,3]. We therefore investigated the possibility that the mRNA for PrP^c is differentially expressed in the suprachiasmatic nuclei, the principal site for the generation of mammalian circadian rhythms. Adult male Wistar rats maintained in a 12:12h light:dark (LD) cycle (lights on was designated as Zeitgeber time [ZT] 0) were used. To establish whether the levels of mRNA for PrP^c change across this cycle, rats were sacrificed at ZT 0, 2, 6, 10, 12, 14, 18 and 22. In order to determine if there is a variation in this mRNA in the absence of LD entrainment cues, lights were not turned on at the usual time (designated as circadian time [CT] 0); the animals were killed on day 2 of constant darkness (DD) at CT 0, 2, 6, 10, 12, 14, 18 and 22. *In situ* hybridisation histochemistry was carried out on coronal sections taken at the level of the SCN using a PrP^c oligonucleotide probe labelled with ^{35}S. Quantitative image analysis of the autoradiographs at the mid-rostrocaudal level of the SCN revealed a significant ($p<0.001$, $n=5$ at each time point) temporal variation in the signal for PrP^c mRNA in the SCN and in other sites examined i.e. supraoptic nuclei, caudate putamen, cingulate cortex, parietal cortex and piriform cortex. This variation was observed not only during the LD cycle but also in DD. Nevertheless, at all of the sites examined the peak signal occurred two hours into either the real or the subjective night i.e. at ZT 14 or CT 14, respectively. The peak at this time point in LD and DD follows the onset of behavioural arousal after ZT 12 or CT 12. To test the hypothesis that the rise in PrP^c mRNA might be a consequence of increased arousal, animals were kept awake and ambulatory by continuous handling between CT 8 and CT 10; northern blots involving the use of the same oligonucleotide probe were used to detect PrP^c mRNA in the whole forebrain of these animals and of other animals killed at CT10 or CT 14 without prior handling. A significant rise ($p<0.05$, $n=5$ at each time point) in PrP^c mRNA between CT 10 and CT 14 was observed in the forebrain of the non-handled rats; this indicates that the increased expression found using *in situ* hybridisation histochemistry is not restricted to regions in the same coronal plane as the SCN. The animals that were handled continuously between CT 8 and CT 10 showed no change in the expression of PrP^c mRNA at CT 10; this suggests that increased arousal is not a significant factor underlying this widespread and synchronous peak of PrP^c mRNA expression. To our knowledge, this is the first study to demonstrate that the expression of prion protein mRNA can change over a relatively short period *in vivo*.

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Phase, amplitude and kinetic differences in the rhythmic expression of two zebrafish BMAL1 homologs

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PAS-domain-containing proteins, and in particular bHLH-PAS factors, are a common feature of molecular clocks in various model organisms. Clock, a gene encoding a bHLH-PAS transcriptional activator, was first isolated through a genetic screen in mouse, and then by homology in Drosophila. Recently, the zebrafish homolog of mouse Clock was shown to oscillate independently in many tissues. We sought to look for zebrafish Clock (zfClock) partners using a two-hybrid system approach. cDNAs encoding two different proteins were isolated. Both proteins are similar to mammalian protein BMAL1, which has been the only partner of Clock identified so far. This is the first case of two different partners of Clock isolated from one organism. In order to see if these two zebrafish BMAL homologs (zfBMAL1 and 2) fulfill the same role or have distinct functions, we compared their expression pattern in various tissues and conditions. The zfBMAL transcripts oscillate in almost every tissue examined. However, in many tissues, the peak, the level and the kinetics of expression are different between both isoforms. Moreover, these parameters change for the expression of each gene from tissue to tissue. These results are consistent with the growing body of evidences pointing towards independent oscillators in various tissues in vertebrates. In addition, it strongly suggests that the two zfBMALs have different functions in the zebrafish circadian clock, perhaps interacting with their partner zfClock at different times in different tissues.

ENCEPHALIC PHOTORECEPTORS IN LOWER VERTEBRATES

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Environmental light passes through the brain, and in non-mammalian vertebrates, is detected by encephalic or "deep brain" photoreceptors.

These photoreceptors play an important role in the regulation of temporal physiology, mediating the entrainment of circadian clocks and photoperiodic events such as seasonal reproduction and migration.

Until recently, the cellular location and the nature of the photopigments utilized by deep brain photoreceptors remained a mystery. In recent immunocytochemical studies we have used antibodies raised against photoreceptor rod and cone opsins, α-transducin and arrestin in an attempt to localize encephalic photoreceptors in teleosts and amphibians.

Several prosencephalic areas were labelled, including a subset of cells in the preoptic nucleus of all species examined. In addition, some species showed immunolabelling in the SCN and in the basal telencephalon. Using in situ hybridization approaches, cells within the subhabenular region of the teleost brain were found to express VA-opsin, a recently isolated "novel" opsin.

Collectivelly, our results strongly suggest that: i.) fish and amphibians possess multiple encephalic photoreceptors; ii.) the photopigments they employ are opsin-based; iii.) at least some of these photoreceptors are located in regions of the brain thought to contain circadian oscillators. These new findings will be compared to our previous results in lampreys and reptiles.

THE NEURAL CLOCK GOVERNING FACIAL MOTOR SYSTEM

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Tremor can be considered not an unwanted byproduct of movement performance, or the exclusive result of the inertial and viscoelastic properties of moving body parts, but a necessary background for coordinated execution of movement. The nictitating/eyelid response has been used for many years as an experimental model for the study of motor learning. A recent study of the kinetic properties of cat upper lid suggests the existence of a ≈ 20 Hz oscillator underlying reflex and conditioned responses. It has been also reported that the increase in the duration of reflex blinks was accomplished by the addition of successive downward waves of ≈ 125 ms, suggesting that the process of reaching a target in a given time is achieved with a fixed-frequency neuronal oscillating machinery. When the available data on lid dominant oscillation frequencies for different species are plotted against species body weight, an inverse logarithmic relationship is obtained, suggesting that lid biomechanics must be tuned to the lid's weight and to its viscoelastic properties. The slope of this relationship (- 0.25) indicates that the lid oscillatory frequency could be related to oxygen consumption per unit body mass.

Since the eyelid is load free, and facial motoneurons receive no feedback proprioceptive signals from the orbicularis muscle, it could be suggested that the oscillatory behavior of the eyelid is the result of the activity of the neuronal mechanisms controlling it. In fact, it has been shown recently in cats and rats that tremor of the lids in an inherent rhythmical property of facial motoneurons innervating the orbicularis oculi muscle. Moreover, a noticiable oscillatory behavior has been observed in cat pericruciate cortex and in cerebellar interpositus neurons during reflexively evoked and conditioned blinks. Coherent 25- 35-Hz oscillations have also been reported in the sensorimotor cortex of awake monkeys during exploratory and manipulative movements.

Taken together, these data suggest that motor systems could be controlled by central neural clocks tuned to the inertial and viscoelastic needs of moving appendages.

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Biochemical and functional analysis of mammalian photolyase homologs CRY1 and CRY2

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In search for mammalian homologs of the DNA repair enzyme photolyase, we have cloned from mouse cDNA libraries two genes belonging to class I photolyases: *mCry1* and *mCry2*. Using immunofluorescence microscopy with either CRY1 or CRY2 specific antibody or CRY-GFP fusion proteins, we found in mouse liver cells as well as in several mouse and human cell lines that CRY1 is located in the mitochondria, while CRY2 is found mainly in the nucleus. This difference in subcellular localization is explained by the presence of a mitochondria transport signal in the N-terminal region of CRY1 and a unique nuclear localization signal in the carboxyl terminal region of CRY2. Endogenous CRY proteins showed an apparent difference in biochemical characteristics. CRY1 binds tightly to DNA Sepharose, while CRY2 is found in the run-through fraction. However, both proteins have no specific binding activity to UV damaged DNA, suggesting that the CRY proteins are involved in other processes than UV damaged DNA repair. To study the functions of CRY proteins, we disrupted the *Cry1* and *Cry2* genes in the mouse germ line and analyzed circadian wheel running behaviour of *Cry* single and double knockout mice. Remarkable differences in the free-running clock became immediately apparent when animals were subjected to constant darkness from normal light/dark cycles. While *Cry1* and *Cry2* single mutant mice exhibit an accelerated and delayed free-running periodicity of locomotor activity, respectively, an instantaneous complete loss of rhythmicity is observed in double knockout mice under constant darkness. These results demonstrate that both CRY proteins are essential for maintenance of circadian rhythms.

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LIGHT-INDUCED FOS EXPRESSION IN THE RD MOUSE RETINA

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Mice homozigous for the allele *rd* (retinal degeneration) are frequently used in studies about circadian biology because they still maintain their ability of synchronisation to the external light-dark cycle despite the fact that they are completely blind for visual responses. This mutation causes a massive dead of rods followed by a loss of cones. At three months of age *rd* retina show only a few rods and a small number of cones located exclusively within the dorsal half of the retina. At one year, there is only a few cells expressing cone opsin in the dorsal retina. However these cells lack outer segments and there are doubts about their ability to respond to light. In the retina Fos expression can be use as marker of light responses. Since opsin expression in rd mice decrease with age and is faster in the ventral than in dorsal retina, in this work we will use light induced Fos expression in the retina to know whether this expression correlates or not with the presence of opsin. We have found that:

1) light-induced Fos expression in the retina is lower in the *rd* retina than in the wild type retina, and that in the mutant retina it decreases in the first three months of life. After that, light induced Fos expression remains at the same levels,

2) there are the same number of cells expressing Fos in the dorsal half of the retina (where there are cells expresing opsin) as in the ventral half.

These results suggest that light-induced Fos expression in the *rd* retina may be due to a pathway of light perception independent of the classical rod and cone photoreception.

Mammals employ novel photoreceptors in regulation of temporal physiology. Robert J Lucas, Melanie S Freedman, Russell G Foster.

Abstract. In an attempt to identify the ocular photoreceptors which are responsible for regulating the mammalian circadian system, we have generated two separate strains of mice (*rdta/cl* and *rd/cl*) in which both rod and cone photoreceptors have been targeted for ablation. Detailed immunocytochemical, northern blot and rt-PCR analysis has confirmed that these animals lack classical rod and cone photoreceptors. Nonetheless our results indicate that upon exposure to monochromatic 510nm light, these mice are capable of exhibiting both pineal melatonin suppression and phase shifts in circadian activity rhythms in an irradiance dependent fashion. We did not find any indication of attenuated sensitivity in these mice. Our results indicate that mammals are capable of employing as yet unidentified novel photoreceptors in regulation of temporal physiology.

CIRCADIAN RHYTHMS OF GLUTAMATE, GABA AND TAURINE IN THE NEOSTRIATUM, NUCLEUS ACCUMBENS AND PREFRONTAL CORTEX OF THE AWAKE RAT. B. Márquez de Prado, A.Galindo, T.R. Castañeda, A. Del Arco, G. Segovia, F. Mora*. Dept. of Physiology, Fac. of Medicine, Univ.Complutense, 28040 Madrid (Spain).

The aim of the present study was to investigate the circadian rhythms of the extracellular concentration of the neurotransmitters glutamate (GLU) and GABA in the neostriatum, nucleus accumbens and prefrontal cortex of the awake rat. Also taurine (TAU) was measured.

Microdialysis experiments were performed in male Wistar rats during a dark:light:dark (6h:12h:6h) cycle. Samples were collected every 1 hour. Amino acids were analysed by reverse phase HPLC with fluorometric detection.

Extracelullar concentrations of GLU show a circadian rhythm, decreasing with light ($p<0.05$) and increasing in the dark ($p<0.05$), in neostriatum and prefrontal cortex but not in the nucleus accumbens. Extracelullar concentrations of GABA show a dark-ligth rhythm, decreasing with light ($p<0.0000$) and increasing in the dark ($p<0.05$), in neoestriatum but not in nucleus accumbens and prefrontal cortex. TAU does not show circadian rhythmicity in any of the areas studied.

These results suggest that the extracellular concentrations of GLU and GABA but not TAU have a circadian rhythm which is especific of the area of the brain studied.

HAIRY GENES IN VERTEBRATE SEGMENTATION AND SOMITOGENESIS.

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Trunk segmentation in vertebrate embryos occurs progressively: groups of cells bud off from a terminal growth zone, the unsegmented presomitic mesoderm (PSM), to form somites which give rise to a variety of segmented tissues. Recent experiments in chicken embryos done in collaboration with our laboratory (Palmeirim et al., Cell 91, 639-648: 1997), provide the first direct evidence of a molecular clock linking segmentation and somitogenesis. In that work, it was shown that cells of the PSM express *hairy1* transcripts in a series of pulses whose 90 min periodicity corresponds to that of somitogenesis. A similar pattern of cyclic expression in the PSM was described for *lunatic fringe* in chicken (McGrew, M.J. et al, Curr. Biol. 8, 979-82:1998) as well as in mouse embryos (Forsberg, H. et al., Curr. Biol. 8, 1027-30: 1998).

In order to determine if cyclic *hairy1* expression is controlled at the level of transcriptional initiation or by the stability of its messanger RNA, we have measured rates of transcription *in vivo*. We used *in situ* hybridisation with a *hairy1* intron probe to visualise nascent nuclear transcripts in the PSM. We find that the expression pattern of unprocessed nuclear *hairy1* transcripts is like that of cytoplasmic *hairy1* mRNA. This argues that the cyclic accumulation of *hairy1* transcripts is due to periodic initation of transcription.

We are currently trying to define minimal promoter fragments that drive cyclic transcription in the PSM ("clock-elements") by analysing reporter constructs in transgenic mice. Genomic cosmid clones from various genes that cycle in the PSM, including *c-hairy1*, *lunatic fringe*, *c-hairy2*, and murine *HES1* (which is homologous to *c-hairy2*), have been isolated and the promoters of *Hes1* and *hairy1* have been partially sequenced. The *Hes1* promoter is being analysed by testing the ability of fragments to drive expression of a *lacZ* reporter gene in the PSM of 8.5-9.5 dpc mouse embryos. Because reporter *lacZ* transcripts and protein may be too stable to visualise cyclic expression, we have also generated a modified reporter construct in which an heterologous intron upstream of the *lacZ* coding sequences will allow us to monitor nascent transcripts.

Serotonin, light and the suprachiasmatic nucleus

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Serotonergic neural pathways play an important role in the phase-shifting effects of light on the circadian system in the rat. This statement contrasts with the general view that excitatory amino acids, acting directly or indirectly through the retino-hypothalamic tract are the main if not the only neural pathway mediating the effects of light on the circadian system. This controversy is possibly due to the important differences observed among species (ie, hamsters and rats) and the still unexplained differences observed and reported between in vitro and in vivo experiments utilising serotonergic drugs within a single species.

We wish to present new evidence which supports the importance of serotonin in the mediation of light. Histological sections of rat brain containing SCN were immunostained for the 5HT2a and 5HT2c receptor subtype. We have also constructed a phase response curve of *c-fos* induction by 2mg/kg DOI (full name), a serotonin agonist that acts at the 5HT2a/2c receptor subtypes. Results show that the induction of *c-fos* throughout a 24 hr period mimics the effects of light pulses administered at similar times. We also suggest that this drug is acting through the 5HT2c receptor subtype on account of its relative abundance in comparison to the 5HT2a receptor subtype in the SCN. We believe these results add further support to the already compelling evidence that serotonin plays an important role mediating the transmission of photic information to the circadian system.

Molecular analysis of the *period* gene in the housefly, *Musca domestica*

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The *period* (*per*) gene of *Drosophila melanogaster*, which lies at the core of the fly's circadian clock, controls a number of biological rhythms, including the circadian periodicity of locomotor activity and adult emergence from the pupal case. Levels of *per* gene products cycle with a 24 hour period, as required for a clock molecule. Furthermore, a temporal delay between the peak expression of *per* mRNA and protein suggests the existence of a negative feedback loop through which Per protein regulates the synthesis of its own mRNA.

In an attempt to determine the extent to which clock molecules and mechanisms are conserved among dipterans, we cloned the *per* homologue from the housefly, *Musca domestica*. The *Musca* gene encodes a 1072 amino acid protein in which areas of high similarity with *Drosophila per* are interspersed by non-conserved stretches. Areas of high conservation include the amino terminus, the PAS domain and the region surrounding the per^S mutation site of *D. melanogaster*. Successively we assessed *Musca per* functionality in the *D. melanogaster* circadian machinery. Behavioural analysis of transgenic per^0 fruit flies expressing the *Musca per* homologue (per^0; per^{mml}), demonstrates that the housefly *per* is able to replace endogenous *per* functions in the host's clock system.

Expression of *per* products was investigated in both housefly and transgenic fruitfly. Housefly *per* transcript levels display daily changes in abundance, similar to those observed in wild-type *D. melanogaster*. In sharp contrast, no circadian fluctuations in the amount of Per protein were observed in *Musca*, as analysed by western blotting on total head extracts.

Circadian oscillations of *per* transcript were found in per^0; per^{mml} transformants, even though with a lower amplitude than wild-type. These transformants also display a circadian oscillation in Per protein, albeit no temporal delay was observed between the transcript and the protein peaks. The possibility exists that the exogenous Per protein undergoes altered modification / degradation events in a wild-type host. per^0; per^{mml} could therefore become a useful model to study this aspect of Per metabolism.

THE CIRCADIAN OSCILLATOR AND OTHER FACTORS INFLUENCE THE RESPONSIVENESS OF *Phaseolus vulgaris* SEEDLINGS TO LIGHT

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The regulation of gene expression and of stabilization of LHCII (light-harvesting apoprotein - the major antenna chlorophyllprotein complex of photosystem II), is performed by light *via* phytochrome action and the biological clock. Light induces the rhythmic expression of several *Lhc* genes and the accumulation of chlorophyll and LHCII apoprotein in a circadian manner. The pacemaker was found to function also in total absence of light, controlling the low levels of expression of *Lhcb1* gene as well as the accumulation of chlorophyll when bean seedlings are exposed to light (J.H. Argyroudi-Akoyunoglou and A. Prombona, J. Photochem. Photobiol. 36, 271, 1996 and Prombona et al., Kluwer Academic, Netherlands, 1998 in press). The stabilization of the mature LHCII protein is governed by a thylakoid bound protease with oscillating activity induced by phytochrome action (Bei-Paraskevopoulou et al., Photosynthesis Research 44, 93, 1995). In order to understand further the function of the central oscillator in plants, the interaction of light and the phytohormone auxin (indole-3-acetic-acid) was investigated. The experiments studying *Lhcb1* transcript and LHCII protein accumulation show that the responsiveness of etiolated seedlings to light (2' white light flash) varies according to the phase of the preexisting rhythm and that auxin treatment accelerates the acute response of the plant to light.

Melatonin blocks the activation of estrogen receptor for DNA binding

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We have observed that melatonin prevents, within the first cell cycle, the estradiol-induced growth of synchronized MCF7 breast cancer cells. By using nuclear extracts of these cells, we have first examined the binding of estradiol-estrogen receptor complexes to estrogen responsive elements, with the finding that addition of estradiol to whole cells activates the binding of the estrogen receptor to DNA, whereas melatonin blocks this interaction. By contrast, melatonin neither affects the binding of estradiol to its receptor nor the receptor nuclear localization. Moreover, we also show that addition of estradiol to nuclear extracts stimulates the binding of estrogen receptor to DNA, but this activation is also prevented by melatonin. The inhibitory effect caused by melatonin is saturable at nM concentration, and does not appear to be mediated by RZR nuclear receptors. The effect is also specific, since indol derivatives do not cause any significant inhibition. Furthermore, we provide evidence that melatonin does not interact with the estrogen receptor in the absence of estradiol. Taken together, these results demonstrate that melatonin interferes with the activation of estrogen receptor by estradiol. The effect of melatonin suggests the presence of a receptor, which upon melatonin addition, destabilize the binding of the estradiol-estrogen receptor complex to the estrogen responsive element.

Zebrafish *Clock* Gene Oscillations Reveal Independent Circadian Pacemakers in a Number of Tissues. Whitmore, D., Foulkes, N.S., Strahle, U., and Sassone-Corsi, P.

The cloning of the *clock* gene in the mouse, following a large scale mutant screen, marked the identification of the first circadian clock gene in a vertebrate. By use of a low stringency cDNA library screen we cloned a zebrafish homolog of the *clock* gene, which showed a high degree of similarity to the mouse counterpart. Data collected using RNase protection revealed that the *clock* transcript oscillates with a pronounced circadian rhythm in the eye and pineal gland, the two established circadian pacemaker structures in the fish. This is in sharp contrast to the situation in the mouse, where *clock* expression shows no day-night changes. Further examination revealed that *clock* mRNA also oscillates in many tissues within the fish when dissected at a variety of times across the circadian cycle. This observation raised the possibility that each tissue may contain it`s own circadian oscillator, or that these peripheral oscillations are driven from a central "master" clock. To answer this question we placed several tissues into culture in constant darkness, and, at a number of phases, RNA was extracted and the level of *clock* transcript was assayed. The oscillation of *clock* observed in vivo was also clearly apparent in vitro, in the absence of any timing cues. We can conclude that the heart and kidney of the zebrafish do, in fact, contain an endogenous circadian oscillator.

In this poster we present the data described above, as well as new information regarding the entrainment of these peripheral tissue clocks, and the development of the circadian clock in the zebrafish embryo.

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