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The Role of Hypothalamic Circadian Rhythms in Regulation of Reproduction

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Much attention is paid in the literature to the role of diurnal rhythms of biogenic amines' hypothalamic contents in regulation of many functions of an organism, among which are motor activity and behavior, sleep-awakening cycle, endocrine and immune systems. The number of studies of reproduction regulation mechanisms participating in circadian signal transduction from the suprachiasmatic nuclei to hypothalamic structures responsible for gonadoliberin synthesis and secretion (the preoptic area – PA, and the medial eminence – ME) is presently being rapidly increased. Disturbances of estrous cycle in female rats in ageing have been shown to be accompanied by the dampening of circadian rhythms of the activity of monoaminergic and opioid hypothalamic systems playing a key role in regulation of gonadoliberin synthesis and secretion. The present studies have revealed disturbances of circadian rhythms of biogenic amines' (dopamine, norepinephrine and serotonin) contents and the rate of reactive oxygen species' formation in hypothalamic structures responsible for reproductive cycle regulation affected by a number of neurotoxic agents (toluene, 1,2-dimethylhydrazine). The estimation of those parameters can be useful for an identification of disturbances of hypothalamic mechanisms of reproduction regulation caused by other neurotoxic agents as well as in some diseases and ageing. A decrease in gonadoliberin level and the dampening of biogenic amines' diurnal changes have been found in PA and ME after exogenous administration of pineal gland hormone melatonin that plays an important role in the entraining of biological rhythms of the organism. The data obtained testify to possible negative side effects of melatonin on hypothalamic regulation of reproduction when applied for the therapeutic purposes. This work was supported by RFBR grant No.00-04-48967

[DOI:10.1240/sav_gbm_2003_m_000294]

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Effects of melatonin (mel) on gene expression in human cell lines stably transfected with either MT1 or MT2 mel receptors.

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Mel entrains the master biological clock [the suprachiasmatic nucleus (SCN)] by activation of specific receptors. In mammals two types, MT1 and MT2, have been cloned. MT1 is expressed in the pituitary pars tuberalis and the SCN, i.e., in the presumed sites of the reproductive and circadian actions of mel, whereas MT2 is mainly expressed in the retina. Differences in signaling between both receptor subtypes have already been shown in transfected cells, but mel receptor specific effects on gene expression in human cells have not yet been studied. To identify which genes are up- or down-regulated in response to MT1 or MT2 activation, these receptors were stably transfected separately into a human cell line, allowing for the first time a comparative analysis in a homologous cellular context. RNA for microarray analysis was obtained from both transfected and untransfected cells following overnight exposure to mel. Hybridization of Cy3/Cy5- labelled aRNA probes to human 10k DNA chips revealed different expression patterns between MT1 and MT2 transfected cells. More than 20 independent genes representing various functional classes (apoptosis, cell cycle regulation, cell growth) showed a greater than 2-fold change in expression in a cell line-specific manner. Immunoblotting and real-time PCR are being used for post-array validation. Our data indicates that mel has diverse effects on gene expression depending on the mel receptor subtype involved. [This study was supported by the DFG-GRK 336]

[DOI:10.1240/sav_gbm_2003_m_000372]

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Modeling feedback loops in the mammalian circadian core oscillator

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Analysing a new theoretical model of the circadian oscillator, we observe a strong influence of the positive feedback on the persistence of oscillations. The oscillatory expression pattern of the clock genes is the molecular basis for the circadian rhythm. It results from positive and negative feedback loops, in which the clock genes regulate their own transcription. In a negative feedback loop CRY1 and CRY2 decrease their own transcription by inhibiting the transcriptional activator BMAL1:CLOCK. PER2 in a positive feedback loop activates the transcription of *Bmal1*, which increases the amount of BMAL1:CLOCK and therefore the transcription of *Per* and *Cry* mRNAs.[1] While the delayed negative feedback is necessary for sustained oscillations, the function of the positive feedback is so far an open question. In order to find possible answers, we constructed a simple model of the oscillator, which only contains the main components of the negative and positive feedback, *Bmal1*, *Cry* and *Per2*. With a system of seven differential equations we can reproduce the experimentally observed dynamics of the considered mRNA and protein concentrations. The robustness of the dynamics is examined with respect to parameter variations. We compare the dynamical behavior of the system with and without positive feedback. It turns out that without positive feedback a certain decrease of the negative feedback destroys oscillations. In the presence of the positive feedback oscillations can arise from a decrease of the negative feedback. These simulations provide a possible explanation of the counterintuitive behavior of recently studied double mutants [2]. We conclude that the positive feedback can support the maintenance of oscillations.

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[DOI:10.1240/sav_gbm_2003_m_000379]

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Memory and the biological clock: age-dependent effects on neurochemistry and behaviour

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Memory retention of a conditioned response is influenced by circadian processes. In rats, optimal performance in passive (PSA) and active shock avoidance (ASA) recurs with a period of 24 hours after acquisition with worse retention in-between [1; 2]. We have shown that retention deficits at 18 and 30 hrs after ASA persist in continuous light conditions. This demonstrates their endogenous nature, independent from external LD cycles. Interestingly, no memory deficit occurs in vasopressin (AVP) deficient Brattleboro rats. In aged rats, no memory oscillations exist when testing an individual repeatedly at multiple time points in PSA, whereas they do occur in young rats. These results suggest an inhibitory role for the circadian system in memory retention. We also studied SCN neurochemistry in the rat in response to ASA. Muscarinic acetylcholine receptor (mAChR) density increases to the highest degree 24 hrs after training, with an anticipatory increase at 22 hrs. In contrast, aged rats show no increase in mAChR-density 24 hrs after ASA. Surprisingly, AVP cell count and content were increased in aged, but not young rats 24 hrs after ASA. This suggests uncoupling of cholinergic and AVPergic SCN systems. Our results strongly support the idea of (inhibitory) role for the SCN in - time dependent - memory retention. Since behaviour and neurochemistry are affected independently with ageing, we conclude that a less well organised circadian system influences circadian memory modulation.

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[DOI:10.1240/sav_gbm_2003_m_000386]

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The intergeniculate leaflet (IGL) - in vitro studies.

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In the present study we have performed extracellular recordings of a spontaneous activity of the rat intergeniculate leaflet (IGL) neurons in vitro. This thalamic structure receives a bilateral and overlapping retinal projection which arises from all parts of the retina [1]. In the majority (but not all) of the retinal ganglion cells, glutamate functions as an excitatory neurotransmitter. The effect of L-glutamate and N-methyl-D-aspartate (NMDA) on the discharge activity of neurons in this structure was studied in our laboratory. We have observed that in the standard incubation fluid IGL neurons can display at least three types of firing pattern: irregular, continuously firing neurons with a wide variety of firing rates, tonic firing neurons, with very stable level of activity, phasic (slow bursting) neurons with intermittent silent periods. All substances were applied in pressure injection. Application of L-glutamate induced biphasic response, i.e. an initial transient excitation succeeded by an inhibition, or only an inhibitory response. Application of NMDA induced either an excitatory response (in majority of the examined neurons), or an inhibition. The inhibitory actions of NMDA and the inhibitory component of biphasic response mediated by L-glutamate, are probably induced by an activation of the inhibitory interneurons. These results are the first electrophysiological demonstration of the neuronal activity of IGL in vitro. Our data are consistent with the prevalence of a glutamatergic input to the visual and the circadian system.

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[DOI:10.1240/sav_gbm_2003_m_000368]

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Influence of systemic application of 8-OH-DPAT on the intergeniculate leaflet (IGL) neuronal activity.

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Systemic administration of 8-OH-DPAT (5-HT_{1A/7} agonist) induces phase advance of rodent circadian clocks during the mid-subjective day [1]. This action of 8-OH-DPAT is blocked after the destruction of intergeniculate leaflet (IGL) [2], the source of neuropeptide-Y (NPY) input to suprachiasmatic nucleus (SCN). Similarly, in vivo injection of NPY antiserum into the SCN attenuates phase shift induced by systemic administration of 8-OH-DPAT [3]. All this suggests that IGL is able to mediate 5-HT agonist-induced phase-shifts of circadian rhythms. The present study is aimed to determine the influence of systemically applied 8-OH-DPAT on the activity of IGL neurons. Experiments were performed on the Wistar rats bred in L:D (12:12) conditions. At the beginning of the light phase animals were anaesthetized with urethane and prepared to perform extracellular recording of IGL neuronal activity. After acquiring a baseline activity of IGL cells, 8-OH-DPAT was administered i.p. (1mg/kg) in the middle of light phase. Within a few minutes after the injection significant increase of neuronal firing in IGL was observed and oscillatory nature of this activity was preserved at the same time. Such increase was observed after electrical destruction of dorsal raphe nucleus [4], source of 5-HT innervation of IGL and also there is data showing that systemic administration of 8-OH-DPAT reduces 5-HT release from the raphe originating terminals [5]. Together it suggests that increase of IGL neuronal firing is triggered by disinhibition of cells caused by reduced 5-HT release. At the same time observed increase of IGL neuronal firing strongly confirms that NPY release can mediate phase-shifting action of systemically applied 8-OH-DPAT.

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[DOI:10.1240/sav_gbm_2003_m_000344]

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Computational analysis of the modular structure of rhythm generation in Crassulacean acid metabolism

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Leaves of the Crassulacean acid metabolism plant *Kalanchoë daigremontiana* exhibit a circadian rhythm of whole-leaf CO₂ uptake [1]. The spatio-temporal distribution of metabolic dynamics in the leaf can be monitored by the chlorophyll fluorescence signal, which reveals dynamical pattern formation in the physiologically homogeneous leaf [2]. Computer simulations and data analysis methods from nonlinear dynamics are applied to image data and multivariate timeseries of whole-leaf exchange, to unveil the degree of synchronization and oscillator-interaction involved in overt rhythm generation [3,4]. The results suggest that the observable rhythm of JCO₂ is the product of a multitude of spatially heterogeneous oscillations in the leaf, which themselves emerge from a network of various rhythmic subunits at the cellular level.

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[DOI:10.1240/sav_gbm_2003_m_000390]

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The circadian system of birds: from genes to behaviour

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Birds have the capacity to perceive information about the photic environment by retinal, pineal, as well as deep encephalic photoreceptors and there are at least three neural circadian oscillators involved in the regulation of circadian rhythms at the organismic level: the retina, the pineal gland, and a hypothalamic oscillator. In the house sparrow, a model species in circadian research, the pineal gland plays a major role in circadian time-keeping by encoding time of day as well as time of year and transducing environmental information into a particular melatonin signal. Additionally, a complex spatio-temporal pattern of Per gene expression can be found in the SCN and in the lateral hypothalamus suggesting the presence of one or several hypothalamic clocks. Rhythms of clock genes can also be found in other brain regions, including the tectum opticum and the cerebellum, as well as in many peripheral non-neuronal tissues, including liver, heart, lung, and gastrointestinal tract. Interestingly, the phase relationship of the various clock genes to each other as well as their phase relationship to the light/dark cycle is in variance to what we know from mammals indicating that the principle autoregulatory feedback mechanisms being responsible for the generation of circadian oscillations in birds may differ significantly from that of mammals. These data provide the basis for a better understanding of the hierarchical organisation of the circadian pacemaking system of birds and the role individual circadian oscillators may play in regulating daily and annual rhythmicity of birds.

[DOI:10.1240/sav_gbm_2003_m_000392]

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New tools for analyzing noise-induced phenomena in biological oscillators

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Spatiotemporal stochastic resonance (STSR) is a phenomenon, where the stability of spatial patterns in an extended dynamical system displays a resonance-type dependence on the noise amplitude with the patterns being optimal at intermediate noise level. This dynamical behavior has been found in theoretical systems as well as in biochemical processes, where the noise level has been controlled externally. However, it is an open question how to identify the signature of a spatiotemporal stochastic resonance in a natural system, e.g., in ecology, when the noise amplitude is not known. This question is addressed in the present paper. We provide analysis tools, which allow to reconstruct the noise intensity in a spatiotemporal data set from the data alone. These tools are based on nearest-neighbor considerations inspired by cellular automata and are an appropriate method for detecting STSR, when combined with some measure of spatial order. As a test of our analysis tools, we apply them to sample data generated by theoretical model systems as well as experimental data sets from biology. We show explicitly that without knowledge of the theoretical value of the noise amplitude for those systems displaying STSR the corresponding resonance curve can be reconstructed from the data alone. In addition, the other nonresonant cases are properly identified by our method with no resonance curve being found.

[DOI:10.1240/sav_gbm_2003_m_000381]

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2D-Protein Gels of the Circadian Rhythms in the algae *E. gracilis*

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To study the molecular origin of the circadian rhythm a simple model system is advantageous. We have chosen the unicellular algae *Euglena gracilis* which displays a clear circadian rhythm with a maximum activity during the time of light at day. The algae were cultured at laboratory condition with an alternating twelve hour day (light) and night (dark) time at room temperature. From the synchronized culture (of ~25 l) aliquots (of ~100 ml) were taken at ~1h intervals and analysed by 2-D-IEF-SDS-electrophoresis gels. The polypeptide spots were studied for variation in intensity of their coomassie stain. There are spots that show a quite constant intensity in all aliquots and a few others vary in time. The polypeptide in the spots have not yet been identified.

[DOI:10.1240/sav_gbm_2003_m_000347]

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Autonomic or endocrine synchronization of slave oscillators by the Suprachiasmatic Nucleus

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Recent findings demonstrated that the clock-genes expression in peripheral tissues. Surprisingly, the rhythmicity of the clock-gene expression in peripheral organs dampens slowly after few days in culture in contrast to the SCN (Reppert et al., 2002). So it appears that SCN must periodically re-entrain peripheral oscillators in order to prevent dampening of circadian gene expressions in peripheral organs. The question is how does the SCN send its circadian signal to the periphery? SCN can use different pathways to bring its "timing" information to peripheral organs: hormonal signal, feeding behavior and autonomic pathways (Buijs et al., 2001). Until now, only hormonal pathways and/or feeding behavior were studied. The data obtained through these studies show that these two pathways are able to entrain the liver's oscillator. However, no study has been performed in order to estimate the role of the autonomic innervations. Therefore in the present project, we investigate the role of the autonomic pathways by analysis the clock-gene expression in the liver. Quantification of clock-gene mRNA expressions is performed by Ribonuclease Protection Assay (RPA) for peripheral tissues, and by in situ Hybridization for SCN.

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[DOI:10.1240/sav_gbm_2003_m_000364]

12

Overlapping and specialised functions of LHY and CCA1 in interacting feedback loops of the Arabidopsis circadian clock

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The MYB transcription factors, LHY and CCA1, have been shown to perform overlapping functions in a negative transcriptional feedback loop that is thought to constitute the oscillatory mechanism of the *Arabidopsis* circadian clock (Alabadi et al., 2001; Alabadi et al., 2002; Mizoguchi et al., 2002). In order to further probe the functional redundancy of LHY and CCA1 in the regulation of circadian rhythms, we attempted to rescue the short-period phenotype of *cca1-1* mutant plants by transformation with an LHY minigene. Surprisingly, this transgene failed to alter the period of LHY gene expression in *cca1-1* plants, although it did lengthen period in a null allele of *lhy*, *lhy-11*. We then tested whether differences in the transcriptional regulation of LHY and CCA1 expression might account for this result. As expected, expression of LHY:luc and CCA1:luc reporter constructs was repressed in LHY- and CCA1-overexpressing plants. We therefore anticipated that loss of function of either LHY or CCA1 would reduce the level of negative feedback regulation and lead to increased expression of both reporter constructs; however, expression of CCA1:luc was reduced about 100 fold in the *cca1-1* mutant, and expression of LHY:luc was reduced by approximately 30%. The *lhy-11* mutation also reduced expression of both reporter genes by approximately 30%. These results indicate that LHY and CCA1 can both mediate positive regulation of their own and of each other's expression. Thus, the *Arabidopsis* clock comprises interlocked positive and negative transcriptional feedback loops, similar to those described for the *Neurospora*, *Drosophila* and mammalian clocks. Intriguingly, LHY and CCA1 can substitute for each other's function in either of these loops, but CCA1 plays a dominant role in positive feedback.

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[DOI:10.1240/sav_gbm_2003_m_000383]

13

Melatonin acutely induces *Cry1* and dampens *Per1* in the Pars Tuberalis of the rat: implications for an hypothetical time-measurement model.

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In mammals, genes involved in a core clock mechanism supposed to constitute the keeping mechanism of inner rhythmicity have been discovered. Interestingly, clock genes were found not only in the master clock located in the suprachiasmatic nuclei (SCN) but also in peripheral tissues where they may interact in a way similar to what happens in the SCN. These tissues however, since they do not require external signals to maintain or express rhythmicity, are rather oscillators than true timekeepers. Among peripheral tissues, the pars tuberalis (PT) of the pituitary expresses clock genes such as *Per1*. It was also demonstrated that, in the PT, melatonin is directly implicated in the control of the expression of *Per1*. As melatonin rhythm depends on the SCN, the PT appears as an interesting tissue to study how the master clock may indirectly entrain a putative oscillator. We show in this study that the PT of the rat, like other rodents, rhythmically expresses *Per1* with peak levels in the early morning. We also observe rhythmic expression of another clock gene, *Cry1*. The endogenous peak of *Cry1* occurs during the night at a time when melatonin level is known to rise. Furthermore, an acute melatonin injection at the end of the subjective day leads to an immediate surge of *Cry1* and a decrease of the *Per1* peak. These data support the existence of a melatonin-driven clock gene expression in the PT and suggest a time-measurement model in the PT based on direct opposite actions of melatonin on *Per1* and *Cry1* expression. Further experiments are in progress to confirm and precise this hypothesis.

[DOI:10.1240/sav_gbm_2003_m_000329]

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ELF4 is required for maintenance of circadian function

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Photoperiodic responses in *Arabidopsis thaliana* require robust interactions between the phototransduction and circadian systems. The mechanisms used for this coordination are unknown. Studies of early-flowering mutants insensitive to photoperiod have led to the discovery that several of these mutants are defective in components of the circadian machinery. I have used this criterion to isolate the circadian mutant *early flowering 4 (elf4)*. It has altered photoperiodic rhythms of gene expression and defects in the overt photoperiodic responses of hypocotyl elongation and flowering time. On one hand, ELF4 functions in the light-input pathway to the clock, as the *elf4* mutant is less sensitive to light. But, the cloning of the *ELF4* gene and resulting molecular analyses have revealed that ELF4 action is regulated by the circadian system. Thus, ELF4 works within a feedback loop between the clock and phototransduction pathways. This connection is highlighted by an interesting phenotype of *elf4*. Circadian rhythms in *elf4* persist under free running conditions, but the ability to properly regulate period length is severely impaired. In fact, individual *elf4* plants deviate from a wild-type period in an unpredictable phase, with both long and short period *elf4* individuals present within an assay. This aspect of the *elf4* phenotype is amongst the first described for circadian mutants of any organism. The collective data leads me to conclude that *ELF4* represents the first member in a class of clock genes, one that functions in maintaining clock accuracy.

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[DOI:10.1240/sav_gbm_2003_m_000382]

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Individual Variation in the Tidal Rhythm of Emergence of the fiddler crab *Uca lactea annulipes*, at a Shore in Kuwait.

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Studies dealing with rhythms of organism in the constant condition of laboratories have a tendency to produce noisy data, for instance variation in the frequency and period length of a rhythm. This study investigates individual variation due to age and sex as one of the possible sources of such variation in the intertidal habitat. Method: The timed behaviour of emergence of the intertidal crab *Uca lactea annulipes* was monitored by video cameras mounted vertically to film 0.5X0.5m.; at an intertidal shore in Kuwait City, Kuwait. The sampling was done during daylight hours, from March 1997, until August 2000, for 3-4 day periods around the new and full moons. The individuals could be easily sexed due to the extreme sexual dimorphism of the enlarged claw of the male. Adult fiddler crabs continue to grow throughout their life, and the larger crabs are older than the smaller ones, therefore the body size could be used as an index of age. The Frontal Breadth of the carapace is used as an indicator of the body size. Results: As the tide recedes the smaller/younger individuals emerge to the surface earlier than the larger/older ones (N= 820, Spearman Rank Correlation Coefficient, P=.0001). The median body size of individuals emerging 1) 3.0-3.5 hours prior to low tide is .6 cm; 2) 2.5-3.0 hours prior to low tide is .7 cm.; 3) 2.0-2.5 hours prior to low tide is .8 cm; 4-5) 1.5-2.0 hours prior to low tide is .9 cm. The males emerge earlier than the females (N=277 Females, Median emergence 2.01 hours prior to low tide, N=543 Males, Median emergence 2.35 Hours prior to low; Mann Whitney Test of Variance, P

[DOI:10.1240/sav_gbm_2003_m_000376]

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Phase Response Curves of Circadian Oscillators

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Phase response curves (PRCs) are crucial for the understanding of entrainment of circadian oscillators to an external zeitgeber. Simulation of models for circadian oscillators allows a systematic study of PRCs. Properties of these theoretical PRCs can be linked to properties of the underlying model, in particular to the way how light stimuli enter these models. Experimentally derived PRCs can be used to validate these theoretically predicted PRCs. From this comparison possible light input mechanism can be revealed. In *Drosophila* light input leads to a rapid degradation of the TIM protein [1]. In mammals discussion focuses on a light induced increase of the transcription rate of *per1* mRNA [2]. Alternatively light might influence the degradation of the BMAL1 protein [3]. We computed PRCs based on simulations of simple models for the circadian oscillators in *Drosophila* [4] and mammals. Different light input mechanisms are simulated and the results are compared to experimental data. We discuss possible reasons for discrepancies between simplified models and experimental data.

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[DOI:10.1240/sav_gbm_2003_m_000378]

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A novel screen for mutants showing defective circadian thermoreception in *Drosophila melanogaster*

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Besides light as a well studied zeitgeber, temperature represents another predominating time-cue. Appearance of light in nature is closely connected with an increase in ambient temperature. In *Drosophila*, there is little known about the receptors and pathways of circadian thermoreception. Rhythmic changes in ambient temperature, differing only in 3°C, are able to synchronize *Drosophila*'s locomotor behavior in constant darkness (Wheeler et al. 1993) and temperature cycles (25°/20°C) in DD furthermore induce circadian oscillations of PER- and TIM-proteins, likely building the base for this behavioral entrainment (Stanewsky et al. 1998). Flies, carrying a *period-luciferase (per-luc)* construct show nicely synchronized luminescence rhythms in 10/14 hours 25°/17°C in DD. In order to identify genes involved in temperature entrainment, chemically mutagenized *per-luc* lines were screened for abnormal bioluminescence rhythms in temperature cycles and DD. So far one mutated line could be found that shows a temperature specific synchronization defect. Mapping of the mutation and further analysis is in progress. Further, our experiments show that temperature synchronization of molecular oscillations can be observed in cultures of isolated Bodyparts, similar as reported for LD-cycles (Plautz et al., 1997). This result suggests the existence of either a cell autonomous circadian temperature receptor - like CRY in light-input - or a signal transduction cascade from a general thermoreceptor to the clock-gene expressing cells.

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[DOI:10.1240/sav_gbm_2003_m_000348]

18

Localization of serotonergic receptors mediating photic-like effects of quipazine

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The mammalian circadian pacemaker in the suprachiasmatic nucleus (SCN) receives dense serotonergic projections both directly from the median raphe nucleus and indirectly from the dorsal raphe nucleus via the intergeniculate leaflet (IGL). In hamsters, serotonergic input to the SCN seems to be involved in the phase-resetting effects of non-photoc stimuli. In contrast, recent studies performed in our lab demonstrate that in rats serotonin (5-HT) agonists induce photic-like effects. For example, microinjections of various 5-HT agonists, such as quipazine or 5-CT, close to the SCN induce photic-like phase shifts of locomotor activity as well as c-Fos expression in the ventrolateral area of the SCN during subjective night, but not during subjective day. Recently, we have started to further characterize the precise localization of the 5-HT receptors mediating the photic-like effects of quipazine. For example, 5-HT could reduce the release of inhibitory transmitters such as NPY or GABA by binding to 5-HT_{1B} receptors located on synaptic terminals of the geniculo-hypothalamic tract (GHT). Lesions of the IGL were thus performed in order to eliminate all projections from the IGL to the SCN. However, preliminary results suggest that IGL lesions do not abolish the photic-like effects of quipazine. Therefore, additional experiments are required to further understand the role of 5-HT receptors within the rat circadian system. Sponsored by DFG (WO354/11-3)

[DOI:10.1240/sav_gbm_2003_m_000312]

19

Activation of the thermosensitive *shibire*-allele *shi^{ts1}* in PDF-expressing neurons slows down the locomotor activity rhythm of *Drosophila melanogaster*.

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In *Drosophila melanogaster* mutants the neuropeptide pigment-dispersing-factor (PDF) plays an important role in the output pathway from the circadian clock to overt rhythms (1, 2). PDF appears to be secreted in a circadian manner into the dorsal brain (3) and transmitted to downstream neurons via G-protein coupled receptors (4). The precise mechanism of rhythmic PDF release is, however, not fully understood. Release seems not to occur via regular synapses involving Synaptobrevin, since tetanus-toxin expression in the PDF-neurons has no apparent effect on the activity rhythm (5). We aimed to analyse another crucial step in synaptic signalling – the reuptake of neurotransmitters that stops signalling. Dynamin is a GTPase protein that is essential for Clathrin mediated endocytosis of synaptic vesicle membranes. The *Drosophila* Dynamin is encoded by the *shibire*-gene (*shi*). We expressed the thermo sensitive mutant allele *shi^{ts1}* in the PDF-neurons to block the constriction of endocytotic vesicles (via the UAS-GAL4 system). This made it possible to "switch" the Dynamin-encoding gene from functioning to non-functioning by upregulating of the housing temperature to 28°C. Meeting this, the flies showed a significant period lengthening of the locomotor activity rhythm by about one hour. If Dynamin regulates the reuptake of PDF into the PDF-neurons, then impairing this endocytosis should extend the presence of PDF in the synaptic gap, and by this means prolong the effect of PDF on downstream neurons, leading to a period lengthening in the activity rhythm. This is in line with the finding that ectopically expressed PDF in the dorsal brain also leads to a longer period (2).

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[DOI:10.1240/sav_gbm_2003_m_000311]

20

A general method for the covalent labeling of proteins in living cells

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Characterizing and monitoring the movement, interactions and life cycle of a protein inside the living cell is the key towards a detailed understanding of its function. Most strategies that aim at realizing this objective are based on genetically fusing the protein of interest to a reporter protein that monitors changes in the environment of the coupled protein. However, all these techniques have various limitations and there are considerable efforts to specifically label proteins in vivo with small synthetic molecules capable of probing and modulating their function. Up to now, these approaches are based on the non-covalent binding of a small molecule to a specific binding protein. We present here a general method for the covalent labeling of fusion proteins in vivo that may open up new ways of studying and manipulating proteins in living cells (1). The covalent attachment of the small molecule to the fusion protein is achieved through the unusual mechanism of the human DNA repair protein O₆-alkylguanine-DNA alkyltransferase (hAGT), which irreversibly transfers the alkyl group from its substrate, O₆-alkylguanine-DNA, to one of its cysteine residues (2). In this contribution we show that hAGT fusion proteins can be labeled with small molecules in vitro and in vivo in different hosts.

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[DOI:10.1240/sav_gbm_2003_m_000357]

21

Isolation of circadian mutants in *Arabidopsis thaliana*: Timing of the induction of *cab* (*tic*) is involved in the gating mechanism.

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Like many organisms, plants have an endogenous 24-hour (circadian) clock to synchronize development and metabolism to the day/night cycle. For example, in *A. thaliana* the *chlorophyll a/b binding protein gene* (*CAB*) is transcribed in a circadian manner. The acute response of *CAB* induction to a light pulse is also regulated by the circadian clock: termed as "circadian gating". Here, to isolate mutants in novel aspects of clock function such as a circadian gating, we generated an EMS-mutagenised population of *A. thaliana* plants carrying the *cab2* promoter-luciferase gene fusion (*CAB2::LUC*). Using a Packard *Topcount* we have isolated 29 mutants, including *gi*, *toc1* or *ztl* allele, from approximately 15,000 seedlings screened so far. *tic* was identified as a low amplitude mutant in constant light. *tic*, like *elf3*, exhibits light conditional arrhythmia and aberrant circadian gating. However, in contrast to the *elf3* mutation which abolishes rhythmicity in both *CAB2::luc* expression and leaf movement in light, *tic* does not have a strong influence on leaf movement rhythms. Flowering time of *tic* was insensitive to photoperiod as another indication of circadian dysfunction. Current progress on the *tic* and other mutants will be discussed.

[DOI:10.1240/sav_gbm_2003_m_000373]

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Rhythms and Oxidative Stress

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Temporal order, as generated by circadian systems, seems to be important for minimizing oxidative stress. Antioxidant enzymes and protective low molecular weight compounds have been repeatedly shown to exhibit circadian or light-controlled rhythms. In the dinoflagellate *Lingulodinium polyedrum*, superoxide dismutase, glutathione S-transferase, hydrogen peroxide destruction capacity, formation of the antioxidants indole-3-pyruvate, kynurenic acid and melatonin showed rhythms of substantial amplitude. Correspondingly, a rhythm in oxidative protein modification (protein carbonyl) was detected. In the short-period mutant *per^S* and in the arrhythmic *per⁰* of *Drosophila*, protein carbonyl was considerably elevated. Interestingly, other investigators found mice being deficient in the homolog *mpcr2* gene to be cancer prone (Fu et al., 2002). Increased protein carbonyl was also measured in the Harderian gland of the Syrian hamster short-day mutant *tau*, which additionally exhibited compensatory rises in superoxide dismutase, glutathione reductase, and catalase. In *L. polyedrum*, oxidative stress by hydrogen peroxide, paraquat or buthionine sulfoximine strongly decreased melatonin; sublethal stress depressed the amplitude of the overt circadian glow rhythm, an effect reverted by melatonin. Oxidative stress may, therefore, also affect temporal order via melatonin destruction.

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[DOI:10.1240/sav_gbm_2003_m_000318]

23

Molecular analysis of the photoperiodic response of flowering in a short-day plant, *Pharbitis nil*

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Photoperiodic response of flowering is mediated by the interaction between environmental light signals and the circadian clock. Recent molecular studies using the long-day plant *Arabidopsis* have demonstrated that the promotion of flowering under long days is caused by the post-transcriptional activation of a clock output gene *CONSTANS* (*CO*) by light, which leads to the transcriptional induction of a flowering time gene *FLOWERING LOCUS T* (*FT*) specifically under long days. To compare the molecular mechanism that controls the photoperiodic response of flowering between *Arabidopsis* and *Pharbitis nil*, a short-day plant, we isolated *FT* related genes from *Pharbitis*. Expression analysis demonstrated that *PnFT1* and *PnFT2* are induced under short days but not under long days, indicating that photoperiodic control of *FT* and *PnFT* is reversed. *PnFT* exhibited a circadian rhythm in constant dark but the rhythms were dampened in constant light, so the oscillation of *PnFT* requires dark. Expression of *PnCO*, a *CO* homolog of *Pharbitis*, was also induced under short days, indicating that different mechanisms control *CO* and *PnCO*, respectively. *PnCO* showed a circadian rhythm in constant dark, and the phase of the rhythm was delayed by entrainment in longer daylengths. Furthermore, *PnCO* levels were rapidly down-regulated by exposure to light. These results are consistent with a hypothesis that the short-day induction of *PnCO* is mediated by a circadian clock function that lacks a *zeitnehmer* mechanism, with its continuous resetting leading to the continuous suppression of *PnCO* during the light. Such a photoperiodic response of *PnCO* might be the cause of the induction of *PnFT* and flowering under short days in *Pharbitis*.

[DOI:10.1240/sav_gbm_2003_m_000330]

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Characterisation and quantification of clock genes in birds

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Birds are equipped with a complex circadian pacemaking system consisting of clocks in the pineal gland, the retina as well as in the hypothalamus. To investigate whether particular clock genes, which are central to the molecular clock mechanism in mammals, are present in central as well as peripheral tissues of the house sparrow (*Passer domesticus*), we cloned *Per2* (96% homologous to quail, 77% to mouse), *Per3* (94% to quail, 82% to mouse), *Cry1* (97% to quail, 96% to mouse), *Bmal1* (97% to mouse, 100% to chicken) and *Clock* (98% to quail, 95% to mouse) in this species. Cloning of *Cry2* revealed at least 4 subtypes. Various central nervous tissues (brain - dissected into telencephalon, diencephalon, tectum opticum and cerebellum, as well as retina and pineal gland) and peripheral non-neuronal tissues (liver, spleen, kidney, muscle, heart, lung and gastro-intestinal tract - dissected into gizzard, duodenum, small intestine and large intestine,) were obtained at eight different time points during LD 12/12 hrs. Tissues were homogenized and total RNA fractions were isolated, reverse transcribed and the resulting cDNA was subjected to RT-PCR. For each set of primers, a magnesium titration was performed and the optimal annealing temperature as well as the optimal cycle numbers for semi-quantitative analysis was determined. The PCR products were subjected to gel electrophoresis, stained with SYBR Green and quantified with a Gel-Doc imaging system. All clock genes were found in all tissues investigated. Levels of clock gene mRNA varied considerably between genes, tissues and time points. These data indicate that molecular circadian oscillations are not only present in central nervous structures of the house sparrow but also in a variety of peripheral tissues.

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[DOI:10.1240/sav_gbm_2003_m_000393]

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Expression of various clock genes in the murine corticotroph AtT-20 cell line

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The generation of circadian rhythms is based on endogenous oscillations within single cells, involving transcriptional and translational feedback loops of clock genes and their products. Notably, these molecular mechanisms are not only present in primary cells of the mammalian master oscillator in the suprachiasmatic nucleus, but can also be observed in many mammalian cell lines. Within rhythm generation, maintenance and phase adjustment, transcription factors of the cyclicAMP signaling pathway, like CREB (cyclicAMP response element-binding protein) and ICER (inducible cyclicAMP early repressor), are thought to play an important role. We therefore investigated in a model cell line for cyclicAMP signaling, the murine pituitary corticotroph tumor cell line AtT-20, expression of clock genes *Per1*, *Per2*, *Bmal1b*, *Cry1*, *Cry2*, *Rev-Erba*, *CK1ε*, and *Clock* under basal and stimulated conditions by RT-PCR. All clock genes investigated are present in AtT-20 cells, and exhibit a circadian rhythm in expression upon forskolin stimulation that could be followed for at least up to 68 h. Peak values in clock gene mRNAs match known dynamics within the interacting feedback loops, as demonstrated in the mammalian suprachiasmatic nucleus. Interestingly also *Icer* showed a clock gene-like rhythmic mRNA fluctuation. Our data indicate the presence of a well orchestrated expression pattern of clock genes in AtT-20 cells and imply an involvement of ICER in rhythm generation, and/or in gating of clock controlled genes. *Supported by the DFG*

[DOI:10.1240/sav_gbm_2003_m_000343]

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SURFACE PLASMON RESONANCE SPECTROSCOPY (SPR) INTERACTION STUDIES OF THE CIRCADIAN CONTROLLED TOMATO LHCA4*1 (CAB 11) PROTEIN WITH ITS PROMOTER

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Feedback regulation is an important biochemical mechanism which is also able to direct the circadian timing at the transcriptional level. Independent investigations highlighted a conserved ca. 10 nucleotide motif present in many circadian regulated genes. Two of such nucleotide motifs exist within 119 nucleotides of the *Lhca4*1* promoter from tomato. This promoter fragment was used as a bait in a yeast one hybrid screen and interestingly a clone encoding approximately 50% of the *LHCA4*1* protein was isolated as an interaction partner. The complete *LHCA4*1* protein was heterologously expressed and binding to the 119 bp promoter fragment was demonstrated by surface plasmon resonance spectroscopy (SPR, Biacore). This result allows to postulate an autoregulatory feedback loop involved in expression of the *Lhca4*1* gene.

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[DOI:10.1240/sav_gbm_2003_m_000320]

27

Differential regulation of light induced c-Fos expression in the SCN by mPer1 and mPer2

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Mammalian circadian rhythms are dominated by a central pacemaker residing in the suprachiasmatic nucleus (SCN). This pacemaker is driven by a molecular-genetic feedback mechanism that partly involves two period genes *mPer1* and *mPer2*. Their role in the regulation of circadian light responses is not completely understood. Here we analyse a possible functional difference between the two *mPer* genes in the regulation of immediate light responsiveness within the SCN. Homozygote *mPer1* and *mPer2* knock-out mice were compared with their wild type background strain. They were stimulated using a monochromatic light source that produced a 500 nm, sub-saturating light stimulus for 15 min. We used *c-Fos* expression to quantify the immediate light responsiveness within core and shell structure of the SCN (as defined by a arginine-vasopressin counterstaining). Before the stimulation, the mice were kept under an LD cycle followed by continuous dim red light. Two experimental groups of mice were stimulated at 6 circadian time points either during their first free-running cycle (Aschoff type II experiment) or after 7-9 circadian cycles (Aschoff type I experiment). The results indicate a differential function for the two *mPer* genes in the regulation of circadian light responsiveness which seems to depend on the type of stimulation protocol.

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[DOI:10.1240/sav_gbm_2003_m_000291]

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Period-2 Cycles and 2:1 Phase Locking in a Biological Clock

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Crassulacean acid metabolism (CAM) serves as a botanical model system for the investigation of circadian rhythmicity. In a new set of experiments with the obligatory CAM plant *Kalanchoe daigremontiana* the response to periodic stimulations with temperature pulses has been studied. On the basis of an experimental phase-response curve of net CO₂-gas exchange the effect of periodic stimulation has been simulated using a finite-difference equation. These simulations revealed the locations of two period-2 cycles in the CO₂ uptake of the CAM plant. In subsequent experiments based upon the simulated bifurcation diagram the position and amplitude of one of these cycles were confirmed, while experimental evidence for the second cycle could be found. Possible roles of such dynamics for the functioning of the biological clock are discussed.

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[DOI:10.1240/sav_gbm_2003_m_000377]

29

The dual external coincidence model in photoperiodic flowering of rice

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Plants can recognize seasonal changes of day-length to leave offsprings efficiently. In the external coincidence model to explain photoperiodisms, which was originally proposed by Bünning (1936) and refined by Pittendrigh and Minis (1964), light functions as a zeitgeber to set the photoinducible phase in the circadian rhythms, in addition to having a role as the external signal. We have demonstrated that a plant photoreceptor, phytochrome, confers the photoperiodic control of flowering in rice, a short-day plant (Izawa et al. 2000). We next examined the interaction between phytochrome signals and a circadian clock mediator, *Hd1*, to control floral pathway integrators, *FT-like* genes, in photoperiodic-flowering mutants of rice and have proposed a model reminiscent of the external coincidence model to explain photoperiodic flowering in rice (Izawa et al. 2002). In addition, we have recently cloned a flowering-time QTL, termed *Early heading date 1 (Ehd1)*, and demonstrated that a novel His to Asp phosphorelay signaling cascade plays an important role in photoperiodic flowering of rice (Doi, Izawa et al. submitted). *Ehd1* mRNA expression may be regulated by both acute light response and the circadian clock. Furthermore, *Ehd1* controls *FT-like* genes independently of *Hd1*. Therefore, we propose a new model, termed as 'the dual external coincidence model' to explain photoperiodic flowering in rice.

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[DOI:10.1240/sav_gbm_2003_m_000339]

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Circadian gene expression in marine red macroalgae

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In marine macroalgae, a variety of circadian phenomena comprising rhythms of photosynthesis, growth, cell division and other processes has been described (e.g. 1, 2). Despite of this, a prove of circadian regulation of gene expression was missing so far for these organisms. Analysis of algal circadian gene expression has been carried out only in microalgae (3). To close this gap, we examined several species of marine red macroalgae for the occurrence of circadian rhythms of photosynthetic oxygen evolution and for oscillation of transcript abundance of photosynthetic genes. In algae which display a strong circadian rhythm of photosynthesis like e.g. *Kappaphycus alvarezii*, a commercial important carragenophyte, we were able to detect diurnal as well as circadian oscillation of mRNA abundance of the genes coding for phycoerythrin and for ribulose-1,5-bisphosphat carboxylase oxygenase. However, other red algae, like *Palmaria palmata*, exhibited no circadian rhythmicity of photosynthesis, at least not under the conditions tested. In addition, no oscillation of transcripts of the analyzed genes could be detected in *P. palmata*. Thus, regulation of photosynthesis by the circadian clock seems to differ within the group of red macroalgae.

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[DOI:10.1240/sav_gbm_2003_m_000359]

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Clock gene expression in the suprachiasmatic nucleus and the pars tuberalis of mel_{1b} melatonin receptor knockout mice

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The mammalian pineal gland secretes melatonin only during darkness to regulate various aspects of physiology and endocrinology. Many of these effects are achieved by melatonin acting via the mel_{1a} and and/or the mel_{1b} melatonin receptor present on cells of the hypothalamic suprachiasmatic nucleus (SCN), as the site of the mammalian endogenous clock, and on cells of the pars tuberalis (PT) of the pituitary, as a window for timing cues derived from the brain. Using mel_{1a}-receptor knockout mice (mel_{1a}R^{-/-}), we recently demonstrated that for rhythmic gene expression, the phasic melatonin signal is only of marginal importance in the SCN, while it is central in the PT. Molecular mechanisms of circadian rhythm generation are based on autoregulatory feedback loops, involving clock genes and their products. To further characterise the role of melatonin within circadian signalling, we investigated daily variations of various clock gene products in the SCN and the PT of mel_{1b}R^{-/-} mice, using immunohistochemical analyses. We found that in contrast to our observations made in mel_{1a}R^{-/-} mice, clock gene products can be detected in the PT of mel_{1b}R^{-/-} mice, however with differences in intensity, as compared to wild-type animals. Our data imply a differential impact of melatonin receptor subtypes for circadian rhythms in protein levels of clock genes in the mouse PT. *Supported by the DFG*

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[DOI:10.1240/sav_gbm_2003_m_000336]

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Daily rhythms in clock gene expression in the mouse pineal gland

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In the suprachiasmatic nucleus (SCN), the mammalian master oscillator, a circadian rhythm is generated by transcriptional and translational feedback loops with clock genes and their products as central elements. In order to disseminate circadian information, the SCN uses, among others, a particularly well-understood output system, the pineal gland, where the hormone melatonin is synthesised and subsequently released. In rodents, the nocturnal elevation of melatonin synthesis is gated by cyclic AMP-dependent transcriptional mechanisms. In addition, a cycling expression of transcription factors of the clock gene family, like mPeriod1 (mPer1), and mPer2 has been demonstrated. However, unlike autoregulatory mechanisms in the SCN, clock gene rhythms seem to depend pivotally on the cyclic AMP signalling pathway. To further analyse the presence and the temporal expression pattern of clock genes in the pineal gland, and to decipher molecular mechanisms behind the regulation of these transcriptional active proteins, we used real-time RT-PCR to study the daily patterns of clock genes expression, that are known to be indispensable in SCN rhythm generation. We found a rhythmic diurnal expression for all major clock genes with peak values in mRNA accumulation, not ruling out autoregulatory feedback loops by their protein products. Our data also support, for the mouse pineal gland, the concept of dual properties for clock genes, e.g. rhythm generation and transcriptional control of clock-controlled genes. Supported by the DFG

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[DOI:10.1240/sav_gbm_2003_m_000370]

THE DIURNAL RHYTHMS OF BIOGENIC AMINES IN HYPOTHALAMIC STRUCTURES AND THEIR POSSIBLE ROLE IN REGULATION OF ESTROUS CYCLE IN RATS

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Accurate timing organization of female rat estrous cycles is due to a daily neuronal signal transmitted by the suprachiasmatic nuclei of the hypothalamus (SCN) to gonadolibernergetic neurons of the hypothalamus at a specific time window during the light period of the day [1]. Monoaminergic systems along with peptidergic systems are known to play an important role in the processes of gonadotropin-releasing hormone (GnRH) synthesis in the preoptic area of hypothalamus (PA) and its secretion in the median eminence (ME). The aim of the present study was to investigate whether monoamine systems participate in transmission of the circadian signal from the SCN. Contents of norepinephrine (NE), dopamine (DA) and the main metabolite of serotonin 5-hydroxyindoleacetic acid (5-HIAA) have been measured in the SCN, PA, and ME of female rat hypothalamus by HPLC-ED method under normal light-dark (12L:12D) or constant dark (12D:12D) conditions. Diurnal changes of DA in all the brain structures under study as well as of NE in the PA have been observed with the peak levels at early day time and trough at the late afternoon or night time. Rhythms of 5-HIAA in the SCN have been shown to have contrary changes. Diurnal changes of biogenic amines' levels in the studied brain structures along with rhythms of opioidergic activity in the arcuate nucleus, that have been shown to be controlled by the SCN [2], may be involved in formation of the time window, in the range of which the circadian signal from the SCN lead to preovulatory GnRH surge. This work was supported by RFBR grants No.00-04-48967 and No.02-04-06234

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[DOI:10.1240/sav_gbm_2003_m_000295]

Fission yeast TIMELESS is required for ultradian and circadian clock function

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The *timeless* gene that is a central component of the *Drosophila* circadian clock has arisen from an insect-specific gene duplication. Little is known about the role, in any biological timing process, of the original *timeless* ubiquitous in eukaryotes. The *Schizosaccharomyces pombe* homologue, in contrast to its metazoan counterparts, is not an essential gene and its function can be assessed easily. We tested mutants for effects on the ultradian clock [1] of fission yeast and found arrhythmicity in both null mutants and overexpression strains as well as longer and shorter periods and loss of temperature-compensation in point mutants. The same was found for the circadian clock. Our bioinformatics analysis has shown that all TIMELESS proteins are more closely related to each other than previously recognised. Most of the protein is of alpha-helical structure, with distinct patterns in the N-terminal and C-terminal halves. While there is some structural similarity to the Arm/HEAT repeat families of proteins, we do not find TIMELESS to belong into either of these families as previously claimed [2] but rather to represent a novel type. The structure of TIMELESS suggests it may be part of larger protein complexes. The *S. pombe* gene has previously been shown to be involved in mating type switching and in this role to be interacting with topoisomerase I and *swi3*, a hitherto unidentified gene [3]. We have studied mutants in both genes and have shown that they, too, are required for the normal functioning of the ultradian clock. Chromatin remodelling has recently been proposed to play a crucial role in vertebrate circadian clock [4]. Or results on the fission yeast *timeless*, as well as the observed period lengthening effects of histone deacetylase inhibitors indicate that it plays also a role in the yeast ultradian and circadian clocks and, by implication, may play a more general role in eukaryotic timing mechanisms.

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[DOI:10.1240/sav_gbm_2003_m_000314]

The *C. elegans period* gene is required for developmental, ultradian and circadian timing

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The LIN-42 protein, known to be involved in developmental timing in *C. elegans*, had been proposed to be a *period* homologue, albeit the similarity encompassing only the PAS domain region [1]. Our reanalysis has shown that the protein had been mispredicted; LIN-42 being considerably longer and showing more similarity to vertebrate PER2. In the light of the recent demonstration of circadian rhythms in *C. elegans* [2,3], we wondered whether *lin-42* might be involved in the nematode's circadian clock. A null mutant was found to be arrhythmic and a ts mutant was arrhythmic at 25°C but showed close to normal circadian rhythmicity at 20°C. The ultradian oscillator controlling the defecation cycle was affected in a parallel fashion in these two mutant strains. This indicates that, as in the case of *Drosophila*, the *C. elegans period* gene is involved in biological timing processes on different time scales and with very different characteristics. A preliminary analysis of genes known to interact with *lin-42* in the developmental timing pathway has shown that they all, including the *C. elegans timeless*, are also involved in the control of the defecation cycle. Phenotypes observed in these mutants were shorter or longer periods, arrhythmicity and, most remarkably, more regular rhythmicity. An analysis of the role of these genes in the mechanism of the circadian clock is now under way, making use of recently developed bioluminescence reporters. Results obtained so far are compatible with the hypothesis that the same network of interacting genes and proteins is involved in multiple biological timing processes in *C. elegans*. Considering that his nematode lacks homologues of other components of the insect and vertebrate circadian systems, our results may have important implications for the evolution of metazoan circadian clocks.

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[DOI:10.1240/sav_gbm_2003_m_000315]

Growth and reproductive development of juvenile European hamsters (*Cricetus cricetus*)

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Annual changes in day length affect the development of newborn European hamsters. Hamsters born in spring or early summer have a higher growth rate and show a more rapid reproductive development than hamsters born in the middle of summer or early fall [1]. According to the present state of knowledge, postnatal development of seasonal species depends on both prenatal and postnatal photoperiods (PPs), i.e. photoperiodic information perceived by the mother during pregnancy and by the juveniles after 14 days of age (after opening of the eyes) [2]. The aim of the present study was to investigate the influence of prenatal and postnatal photoperiodic information on the development of juvenile hamsters. Adult hamsters were paired under different constant or natural PPs. Litters were born and raised under either the same PP or transferred to different PPs at 21 days of age. Preliminary results revealed a significant effect of prenatal PPs on body weight at the day of weaning. Hamsters born under short PPs had a significantly higher weaning weight than hamsters born under long PPs. In addition, postnatal PPs had a significant effect on growth rates and reproductive development after weaning. A summer-like pattern of postnatal development with a high growth rate and rapid reproductive development was observed either in animals born and raised under PPs longer than 15 h or in animals transferred from a decreasing or short prenatal PP to postnatal PPs equal to or longer than 14 h. In contrast, a winter-like pattern with a low growth rate and delayed reproductive development was observed in animals born and raised under PPs shorter than 14 h or in animals transferred from a long or increasing PP to PPs shorter than 15 h. (Sponsored by DFG)

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[DOI:10.1240/sav_gbm_2003_m_000324]

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A new method for sleep spindle analysis: circadian and topographic aspects

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Transient EEG oscillations in the sleep spindle frequency range (12-16 Hz) are an essential feature of human non-REM sleep. Sleep spindles originate in the thalamus and are suggested to have a sleep protective function by reducing sensory transmission to the cortex (1). Spectral analysis of the EEG by means of Fast Fourier Transform has revealed frequency-specific circadian modulation of spindle frequency activity (2). In the present study, sleep spindle characteristics along the anterior-posterior axis were analyzed during and outside the circadian phase of melatonin secretion with a new method for instantaneous spectral analysis (Fast Time Frequency Transform) that yields high-resolution spindle parameters in the combined time-frequency-domain. During the phase of melatonin secretion ("biological night"), the number of spindles was increased and spindle frequency was decreased compared to the phase with no melatonin secretion ("biological day"). Intra-spindle frequency variation was lower during the night than during the day. Spindle amplitude during the night was enhanced in the lower spindle frequency range (14.5 Hz). This circadian modulation of sleep spindle characteristics was dependent on brain topography, being maximal in the parietal and minimal in the frontal derivation. These data suggest that the circadian pacemaker promotes low-frequency, high-amplitude and homogenous sleep spindles during the biological night and provide further evidence for a brain-region-specific regulation of sleep spindles, which is modulated by the circadian pacemaker. Research supported by the Swiss National Science Foundation START Grant # 3130-055385.98.1 to CC.

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[DOI:10.1240/sav_gbm_2003_m_000346]

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The Effect of Neurotoxic Xenobiotics and Exogenous Melatonin on Diurnal Rhythms of Biogenic Amines in Hypothalamic Structures Responsible for LH-RH Production.

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Experiments on chronic inhalation of neurotoxic xenobiotic toluene and single subcutaneous injection of 1,2-dimethylhydrazine (SDMH) possessing neurotropic properties have been carried out on female rats to discover their effects on biogenic amines' system in hypothalamic structures (the preoptic area – PA, and the median eminence – ME). Chronic inhalation of neurotoxic xenobiotic toluene dosed at both maximal permissible concentration (50 mg/m³) and limited chronic range (500 mg/m³) resulted in disturbances of diurnal changes of NE and DA contents in the PA and DA in the ME. Both the evening (11 p.m.) and morning (11 a.m.) administrations of SDMH at the dose of 21 mg/kg body weight resulted in disturbances of all the diurnal rhythms observed in control. In some cases only diurnal rhythms phase shift was found out, in others these rhythms of neurotransmitters disappeared entirely. The chronic effect of synchronizing agent melatonin (administered dissolved in drinking water at the concentration of 10 µg/ml, at night during 2 months) on neurotransmitters and their diurnal variations in both hypothalamic structures proved surprisingly to be much alike the effect of toluene. It is suggested that the effect of toluene and SDMH on the content and diurnal rhythms of neurotransmitters in the hypothalamic structures under study is due to their affecting activities of the enzymes of biogenic amines' synthesis, synaptic transmission, melatonin synthesis and secretion rhythms.

[DOI:10.1240/sav_gbm_2003_m_000293]

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Genetrap Screen to Induce Novel Mutants of Circadianly Regulated Genes in *Drosophila melanogaster*

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A *P*-element mutagenesis screen with a construct, that uses the genetrap strategy (Lukacsovich *et al.* 2000) and *luciferase* as a real time reporter has been performed to identify and mutate novel clock controlled genes (*ccgs*). In this construct the reporter gene *luciferase* does not have a promoter of its own, but a splice acceptor site 5' of its start codon. It therefore can only become active if the construct is located inside a gene and the reporter gene *luciferase* is spliced to the mRNA of a trapped gene. Furthermore this formation of a fusion mRNA between the trapped gene and the *luciferase* destroys the normal function of the endogenous gene. The *in vivo* measurement of 600 transgenic fly lines for rhythmical *luciferase* activity so far led to the isolation of two interesting lines. One line shows rhythmical bioluminescence under LD and DD conditions with a period of 12 h, which has not been described so far. As the oscillation is influenced by mutant alleles of the clock gene *period*, such as *per⁰¹*, which destroys the functional clock, or *per^T*, which shortens the period of the molecular clock to 16 h, the trapped gene is under control of the molecular clock. Heterozygous flies of the other line show a, also clock controlled, rhythmical bioluminescence with a period of 24 h under LD conditions. Flies of this line also have a mutant phenotype in locomotor behaviour. 30 % of the homozygous flies are arrhythmic under LD conditions and 81 % under DD conditions.

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[DOI:10.1240/sav_gbm_2003_m_000350]

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Nuclear transport of circadian clock proteins

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Circadian rhythms are driven by endogenous biological clocks that regulate many biochemical and physiological processes with a 24h periodicity. A conserved set of genes define a transcription and translation-based negative autoregulatory feedback loop that comprises the core elements generating circadian rhythmicity. One important level for the generation of a 24h cycle of the biological clock may be the nucleocytoplasmic transport of the circadian clock gene products. In mammals three putative Per homologues (mPer1, mPer2 and mPer3) have been identified. We have examined the nuclear transport of these circadian clock proteins in *Xenopus laevis* oocytes. In this system only mPer1 and mPer2 can enter the nucleus alone, whereas mPer3, which also contains a putative nuclear localisation signal, remains in the cytoplasm. We established an experimental system that allows the formation of the various Per and Cry heterodimers *in vitro*. After injection of the different heterodimers it turns out that mPer3 is imported in the nucleus only in a complex with mPer1. In mapping experiments we can show that the putative NLS in mPer3 is only active in a fragment of mPer3, but it is not used for the import of the mPer1/mPer3 complex. To mediate transport of this heterodimer the basic type NLS and a novel type of nuclear localisation signal in the C-terminus of mPer1 are needed. We also analysed the nuclear export of the circadian clock proteins.

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[DOI:10.1240/sav_gbm_2003_m_000305]

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The indoleamine melatonin affects early steps of the flowering process in the short-day plant *Chenopodium rubrum*

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Melatonin (N-acetyl-5-methoxytryptamine) was originally identified as an animal hormone. Its levels are high at night and low during the day and can therefore convey information about the daily photoperiodic regime. Melatonin was also found in algae and higher plants. We have previously confirmed melatonin presence in the short-day flowering dicotyledonous plant *Chenopodium rubrum*. Melatonin content in *C. rubrum* exhibits a night maximum, resembling the situation in vertebrates and suggesting its role in plant photoperiodism. Therefore we tested the effect of melatonin on *C. rubrum* flowering. Seedlings were grown in continuous light and flowering was induced by a single dark period (12 h for maximal flowering) at the age of 5 days. 100 μ M and 500 μ M melatonin applied 1 h before the beginning of a 12-h darkness significantly reduced the percentage of flowering plants. 500 μ M melatonin inhibited flowering only when applied 3 h before or during the first half of a 12-h dark period. Uptake experiments revealed that exogenous melatonin was taken up rapidly so melatonin should affect some early step(s) of flower induction or evocation. Because melatonin application did not change the characteristics of the circadian rhythm in photoperiodic time measurement, it must control some other process(es). Application of melatonin agonists and related indoleamines demonstrates that the inhibition of flowering is specific for melatonin. Melatonin agonists 6-Cl-melatonin, 2-I-melatonin, and CGP 52608 all reduced flowering when applied 1 h before a 12-h darkness. 5-hydroxytryptamine which might be metabolized to melatonin was also efficient, while 5-methoxytryptamine had no effect. Possible mechanisms of melatonin action will be discussed.

[DOI:10.1240/sav_gbm_2003_m_000332]

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Altered ROS metabolism in *Per2* mutant mice.

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Elevated levels of reactive oxygen species (ROS) in the cells cause DNA and protein damage, thereby directing the cells to apoptosis to avoid cancer (1). ROS production is normally controlled by several detoxification systems in the mitochondria and in the cytosol. These include superoxide dismutase (SOD), catalase and glutathione peroxidase (1). Some of these detoxification systems seem to be under the control of the circadian clock. We investigated the influence of the circadian clockwork on ROS metabolism. We measured biochemical markers for ROS abundance and detoxification in *mPer2* mutants, which have a defective clock (2). *mPer2* mutant mice showed elevated levels of ROS in the brain compared to wild type animals. The aconitase activity, which is decreased with higher ROS abundance, is lower in *mPer2* mutants and the Mn-SOD activity, which increases with enhanced ROS levels, is higher in these mutants. Furthermore, *mPer2* mutants displayed a higher amount of oxidized proteins in the brain compared to wild type animals. These findings indicate that ROS production is elevated in the *mPer2* mice. However, despite ROS overproduction, *mPer2* mutants do not show a higher susceptibility to cell death than wild type mice, as evidenced by histological analysis in brain and peripheral tissues. At the cellular level, primary embryonic fibroblasts from *mPer2* mutants showed a higher resistance to Paraquat, a drug that leads to cell death through elevating ROS production. Interestingly, the percentage of fibroblasts surviving after the treatment with paraquat is higher for *Per2* mutant mice than for WT animals. These findings indicate that the *mPer2* mutant mice have an altered ROS metabolism and a lower susceptibility to undergo apoptosis. This is in line with the recent finding that *mPer2* mutants are more prone to cancer (3).

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[DOI:10.1240/sav_gbm_2003_m_000361]

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Spatiotemporal patterns of the energy metabolism

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Rhythms are a manifestation of life and occur at all levels of biological organisation. They represent one form of biological order in time documented by their participation in time counting processes, i. e. as circadian clocks. Rhythms occur, however, not only in biological but also in chemical or physical systems (1, 2). There it has been shown, that rhythmic reactions can generate patterns, either in the form of traveling reaction-diffusion waves or as stationary patterns of the Turing-type. Nowadays it is established, that also biological systems can generate such patterns, provided that the basic requirements are fulfilled: an autocatalytic reaction (e.g. a rhythmic one) is coupled to transport processes (3, 4). From a biological point of view, the propagation dynamics of the waves are of special interest, because they contain information. Velocity, amplitude, spiral formation and many other properties depend on the energetic state of the system. Hence, rhythmic reactions contain additional order in space and time, which may introduce new levels for biological information processing. We introduce rhythms of glycolysis in a yeast extract on a spatiotemporal level (5) and give first experimental results about possible external control of these phenomena.

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[DOI:10.1240/sav_gbm_2003_m_000313]

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Calbindin expression in the hamster suprachiasmatic nuclei depends on photoperiodic background

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In mammals, the suprachiasmatic nuclei (SCN) of the hypothalamus contain the main circadian clock which controls many physiological and behavioral rhythms. Some evidence have led the hypothesis that two compartments, one rhythmic and the other non-rhythmic, would constitute the SCN functional entity. The dorsomedial part of the SCN, delimited by vasopressinergic neurons, would mainly contains rhythmic neurons in term of clock gene expression. In contrast, ventrolateral part of the SCN, containing vasoactive intestinal peptide and gastrin releasing peptide neurons as well as Calbindin D28k (CalB) immunoreactive cells would constitute the non rhythmic compartment. Indeed, clock gene expression in the ventrolateral subregion delimited by the Calbindin D28k (CalB) immunoreactive cells would be arrhythmic (1). Moreover, a recent study showed that these CalB expressing neurons do not exhibit a circadian rhythm in spontaneous firing rate, whereas the other neurons in this CalB subregion do (2). In the present study, we show that the Calbindin expression depends on the photoperiodic conditions, and that there is a negative correlation between number of CalB immunoreactive cells and day length. In hamster exposed to short photoperiod (i.e. LD6:18 and LD10:14), number of Calbindin neurons was 2 fold increased when compared to hamster exposed to the long photoperiod LD18:6, and 1.5 fold increased compared to hamster exposed to LD14:10. This finding thus reinforces the view that CalB neurons represent a functionally distinct neuronal subpopulation of the SCN, and suggests their importance in seasonal rhythms.

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[DOI:10.1240/sav_gbm_2003_m_000321]

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Circannual rhythms in the European hamster (*Cricetus cricetus*): Demonstration of an annual phase of sensitivity to long photoperiod

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In adaptation to seasonal changes in their habitat, European hamsters show pronounced annual rhythms in their physiology, endocrinology and behavior. For example, gonads are fully developed only in spring and summer and regressed during winter. These annual rhythms persist even under constant photoperiodic conditions (Masson-Pévet et al., 1994) and can thus be called circannual. Nevertheless, regular changes in photoperiod are necessary for the entrainment of the circannual clock. It has been demonstrated, that European hamsters are sensitive to photoperiodic information between May 15th and July 15th when transfer from a long to a short photoperiod induces gonadal regression (Saboureau et al., 1999). The present study investigated whether European hamsters are sensitive also for an opposite change of day length. 24 animals were kept under natural light conditions, but constant temperature until gonadal regression was completed. Starting September 19th, 5 groups of 4 animals were transferred to long photoperiods (LD16:8) every 4 weeks. Our findings clearly demonstrate the existence of an annual phase of sensitivity to long photoperiods between November 14th and the beginning of March. During this phase of sensitivity, exposure to long photoperiods induced gonadal development within 3 weeks. Therefore, synchronization of the circannual reproductive rhythm to the natural year is achieved by two phases of sensitivity to photoperiodic changes: one phase of sensitivity to short photoperiods in late summer and one to long photoperiods in the winter. Sponsored by DFG.

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[DOI:10.1240/sav_gbm_2003_m_000323]

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Chronobiology of *Clusia minor*: circadian oscillations of C3/CAM intermediate photosynthetic behaviour.

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The reversible C3/CAM photosynthesis of *C. minor* offers an alternative model for the study of endogenous rhythmicity of CAM. We tested the dependence of the endogenous rhythms on temperature in C3 and CAM adapted plants. Oscillations in gas-exchange and chlorophyll fluorescence parameters damped fast, independent of temperature or photosynthetic mode. In all cases, CAM adapted plants returned to C3 during exposure to continuous light. Analysis of correlation between CO₂ uptake and stomatal conductance suggests optimisation of carbon gain and water loss. Images of the relative quantum efficiency (rel. ΦPSII) revealed a very homogeneous pattern over the entire leaf. This is in contrast with observation from *K. daigremontiana*, which showed a dynamic of rel. ΦPSII heterogeneity of in continuous light. Differences on the intercellular air spaces between the species should be responsible for different diffusion rates which affects the spatial distribution of rel. ΦPSII. With the use of non-photorespiratory conditions (1% O₂) it was possible to follow the RUBISCO oxygenase activity in continuous light. The activity of PEPC increase RUBISCO oxygenation by competing for CO₂. Oxygenase activity damped over time which indicated a progressive reduction of carboxylation by PEPC, as the plant switched its photosynthetic mode from CAM to C3. The analysis of rel. ΦPSII showed that the highest light energy use occurred when both PEPC and RUBISCO were active, probably as a result of formation and storage of malate and citrate as well as glyconeogenesis. Oxygenase activity and the rel. ΦPSII tended to stabilise around values typical for C3-adapted plants. The spatial structure of rel. ΦPSII showed also strong oscillations and damped to intermediate values.

[DOI:10.1240/sav_gbm_2003_m_000395]

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Melatonin Receptors in Rat Hippocampus: The MT2 Receptor Modulates the Synaptic Transmission in CA1 Neurons in a Diurnal Manner

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Melatonin has been found to influence the excitability of CA1 pyramidal cells in a diurnal manner (Mußhoff et al., 2002). The aim of the present experiments was to investigate effects of melatonin on the transmission at the Schaffer collateral/CA1 synapse. Rats were entrained to a 12/12 hrs LD cycle (ZT0 = lights on). To perform subjective night experiments during the daytime the rats were accustomed to a reversed light-dark cycle for a minimum of 4 weeks. Hippocampal slices were prepared at ZT 3 (day group; n=29) and ZT 20 (night group; n=20) and analysed in a submerged recording chamber. After stimulation of the Schaffer collaterals field potentials (fEPSP) were recorded from Str. radiatum of the CA1 region. Additionally, single cell PCR experiments were performed with isolated CA1 neurons to analyze the expression of transcripts for MT receptors. Melatonin (10 nmol/l) increased the slope of fEPSP in slices of the day group more than 2 times of the initial level. In contrast to this, melatonin decreased the slope of fEPSP in slices of the night group to 50% of the initial level. Both effects of melatonin were suppressed by administration of luzindole (100 nmol/l) and 4-PPDOT (100 nmol/l). In paired pulse inhibition experiments melatonin exerts opposite effects: a decrease in slices of the day group and an increase in slices of the night group. Transcripts for MT1 receptor are not detectable in CA1 neurons. The data indicate that various forms of hippocampal activity are related to circadian time. The action of melatonin on the synaptic efficacy in CA1 is probably mediated by GABAergic interneurons.

MUSSHOFF U., D. RIEWENHERM, E. BERGER, J.-D. FAUTECK, E.-J. SPECKMANN (2002) Hippocampus 12: 165-173

[DOI:10.1240/sav_gbm_2003_m_000374]

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Circadian regulation of the mitochondrial gene expression in plants?

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We investigate transcriptional regulation of mitochondrial-encoded genes by the circadian clock in *Arabidopsis thaliana*. Previous studies revealed that the circadian clock regulates a number of nuclear-encoded genes at the transcriptional level (1,2). In addition, the expression of a number of chloroplast-encoded genes have also been found to be under clock control (3,4). However, it is still largely unknown whether the circadian clock also controls the gene expression in mitochondria. Only few nuclear-encoded mitochondrial genes, such as a photorespiratory gene, serine hydroxymethyltransferase gene, are reported to exhibit the circadian oscillation in mRNA abundance (5). Towards a comprehensive understanding of a potential correlation between the circadian clock and transcriptional regulation of mitochondrial-encoded genes, we perform macroarray analyses using all of the annotated genes encoded in *Arabidopsis* mitochondria (6). Initially, we investigate the diurnal expression pattern by examining the steady-state transcription levels every 4 hours for 2 days in dark/light cycles. Subsequently we test for the transcriptional regulation by the circadian clock through examining steady-state transcription levels every 4 hours for 2 days during the subjective day and night (i.e., continuous light). The contribution of the circadian clock to mitochondrial-gene expression will be discussed from the point of view of mitochondrial activity in relation to day/night energy demands.

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[DOI:10.1240/sav_gbm_2003_m_000338]

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The role of *mcry1* and *mcry2* in a resetting of the circadian clock

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The function of the circadian system is to synchronize physiology and behavior to environmental cues, mainly the light-dark cycle. Entrainment is accomplished by phase advances or delays of the rhythm according to the temporal state of the clock. On a molecular level, there are five families of circadian genes, directly or indirectly regulated by light. Cryptochromes, *cry1* and *cry2*, are crucial for the function of the circadian clock although their expression is not directly regulated by light. Here, we further evaluate the role of *cry1* and *cry2* genes in phase resetting of the circadian clock by quantification of the product of immediate early gene *c-fos* after light pulses. Light-induced Fos expression in the suprachiasmatic nucleus (SCN) is characterized by phase dependence similar to that for light-induced phase shift of locomotor activity. Thus, maximal phase advances or delays correspond with high *c-fos* expression in the ventrolateral part of the SCN and vice versa. In this study we used single knockout *mcry1*, *mcry2*, and wild type mice. Animals received a 15 min monochromatic light pulse (?=500nm) at the External Time 20 and 4 (ExT20, ExT4; Aschoff type II protocol), which respectively correspond with the times for inducing maximal delays and maximal advances of locomotor activity in this species. *mcry1*^{-/-} mice had a high Fos expression in the SCN after light pulse delivered at ExT20 (ZT14) and no Fos expression at ExT4 (ZT22). In *mcry2*^{-/-} mice, the light pulse evoked lower Fos expression at ExT20 compared with ExT4. These results indicate that *cry1* and *cry2* genes are involved different way in a resetting the circadian clock. We further evaluate this finding by constructing a phase response curve for light-induced Fos expression in constant darkness (Aschoff type I protocol).

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[DOI:10.1240/sav_gbm_2003_m_000340]

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Change in expression of the Glutamate Aspartate Transporter GLAST causes severe physiological perturbations in mPer2 mutants

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Many aspects of mammalian physiology and behavior are governed by the master circadian pacemaker located in the suprachiasmatic nuclei (SCN) of the hypothalamus. mPer2 mutant mice are defective in resetting the clock and become arrhythmic in constant darkness. In light-mediated resetting, glutamate plays an important role. Glutamate is released on nocturnal light stimulation at synapses connecting the eye with the SCN via the Retinohypothalamic tract (RHT). Glutamate binds to its receptors on the SCN neuron, thereby activating common signalling pathways. We found that expression of the Glutamate Aspartate Transporter (GLAST), important in clearing glutamate from the synaptic cleft, is affected in mPer2 mutants. Based on this finding we examined the physiological implications of GLAST deficiency and found an impaired uptake of glutamate into the synaptosomes of mPer2 mutants. Furthermore, a cold induced brain injury showed these mutants susceptible to cytotoxic edema. This might be due to the failure of the membrane ion-pump, which is part of GLAST function. This could lead to a failure in depolarization finally resulting in glutamate accumulation thereby mediating neuronal injury and cell death. This disturbed glutamate signaling may explain the impaired clock resetting in mPer2 mutants.

[DOI:10.1240/sav_gbm_2003_m_000292]

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Correlation of Per1 and Per2 genes expression pattern in the SCN and melatonin peak reappearance after an 8h advance of the light/dark cycle

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Recently we demonstrated that in order to control the daily rhythm of melatonin synthesis, the master clock located in the Suprachiasmatic Nucleus of the hypothalamus (SCN) sends a combination of inhibitory and stimulatory signals to the paraventricular nucleus of the hypothalamus, the origin of the multi-synaptic neuronal pathway that controls the sympathetic input to the pineal gland (Perreau-Lenz et al., 2003). Previously, we also showed that the time delay (5 days in control conditions) for the melatonin peak reappearance following an 8h-phase advance of the light/dark cycle is increased by a 2h restricted-feeding (RF) schedule (Kalsbeek et al., 2000). We hypothesised that the localisation pattern of Per1 and Per2 gene expression in the SCN might correlate with the onset or offset of the nocturnal melatonin peak. In order to test our hypothesis, and reveal the subpopulation of SCN neurons responsible for the control of the melatonin rhythm, we will measure for 6 time-points along the light/dark cycle, using non-radioactive in situ hybridisation, the differential localisation of Per1 and Per 2 genes expression in the SCN of control rats (non-shifted) fed ad libitum, of shifted rats (5 days after the shift) fed ad libitum and of shifted rats (5 days after the shift) previously habituated to a RF diet during 3 weeks. In addition, we will measure the melatonin release before and 5 days after the shift, in ad libitum and RF conditions, by microdialysis in the pineal gland.

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[DOI:10.1240/sav_gbm_2003_m_000365]

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Quantitative RT-PCR as an alternative to northern blot and/or RNase Protection Assays

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Since the days of R.J. Konopkas' discovery of period mutants many new circadian clock genes have been discovered. Most of them show circadian regulation at the transcript level. To measure transcription of old and new clock genes quantitatively, one has to analyze the mRNA with classical methods, like northern blot or RNase Protection Assays. Although applying these methods revealed important mechanisms of the molecular clock, they also have some negative features; e.g.: they are extremely labor-intensive and they involve the use of radioactive labelled probes. As an alternative to those methods we tried to reveal the benefits and disadvantages of the quantitative RT-PCR, using a Lightcycler. The data obtained was compared with results gained by RNase Protection Assays. As an example we analyzed the period and timeless genes of drosophila melanogaster.

[DOI:10.1240/sav_gbm_2003_m_000375]

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Wheel access modifies masking to light without effect on circadian synchronization in *Arvicantis niloticus*

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The Nile rat is a predominantly diurnal rodent which can become more nocturnal e.g. when a running wheel is present (1, 2). It is not known how direct effects of light or dark on locomotor activity relate to the expression of the circadian phenotype in *Arvicantis niloticus*. Exp. 1: Eleven male Nile rats were kept in cages with wheels or without wheels in an LD 12:12 h. Masking during the light phase was tested by repeated cycles of 30 min entraining light (1300 lux) and 30 min of light of a defined light intensity (from ca. 2800 lux to complete darkness). Animals with wheel access were less diurnal than without wheel access. With wheels, dark pulses during the light phase resulted in increases in activity, whereas dark pulses resulted in decreases in activity when no wheels were present. Exp. 2: 24 male Nile rats were kept in a regular photoperiod (LD 12:12 h; 2800 lux during L) and a skeleton photoperiod (LDLD 1:10:1:12 h; 2800 lux during L), with and without wheels in a crossover design. Synchronization was unaffected by the photoperiods. Daytime wheel running during the 12 h L phase was much reduced in the Nile rats with wheels compared to the 12 h LD phase of the skeleton photoperiod. Effects in the tests without wheels were less pronounced, but activity was increased during light exposure. In this study, *Arvicantis niloticus* displayed flexible masking responses to dark and light. With wheel access, light reduced activity (or dark promoted it) and without wheel access light increased activity (or dark reduced it). Wheel access changes the degree of nocturnality through modification of the masking response to light without changing circadian phenotype. Supported by Canadian Institutes of Health Research (to N. Mrosovsky).

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[DOI:10.1240/sav_gbm_2003_m_000384]

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The role of the compound eyes in synchronizing the activity rhythm of *Drosophila melanogaster* to light-dark cycles

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Principally, there are two different ways to synchronize the activity of fruit flies to LD-cycles: (1) "real entrainment" via entraining the molecular oscillations of the clock-genes (*per*, *tim*, *Clk* and *cyc*) in the circadian pacemaker neurons, (2) direct stimulation of activity by light via pathways that bypass the circadian pacemaker neurons, also called "masking". In *Drosophila*, masking manifests itself in an immediate increase in activity after lights-on (the lights-on effect) and a repression of activity during darkness. Although masking works independently of the circadian clock, the provoked activity may feed back on the clock and thus reset it (Mrosovsky et al., 1989). Therefore, masking is not only a potent agent to synchronize the flies' activity to the 24-hour day, but it may also indirectly entrain the clock. We found that masking is solely mediated by photoreceptors of the compound eyes: It is absent in eyeless flies and in mutants without functional compound eyes, but present in photoreceptor mutants that lack cryptochrome or the eyelets. Additionally to mediating masking, the compound eyes appear to contribute to real entrainment. This entrainment may occur via the photoreceptor cells R7 and R8 of the compound eyes that project towards a subset of the pacemaker neurons - the large ventral Lateral Neurons (l-LNV). The remaining photoreceptor cells of the compound eyes (R1-6) are not directly linked with pacemaker neurons and may thus mediate masking. We are currently testing this hypothesis.

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[DOI:10.1240/sav_gbm_2003_m_000351]

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The biological clock and its control of glucose homeostasis

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Mammals adapt their activity pattern to the presence of daylight, and to survive it is essential to adapt energy availability to the activity rhythm. Previously, a daily rhythm in plasma glucose concentrations, generated by the suprachiasmatic nucleus (SCN), but independent of feeding, has been shown by our group¹. Currently, we are investigating several possible ways by which the SCN may control daily glucose homeostasis. Because a daily glucose tolerance rhythm has been shown previously², we are measuring daily variation in glucose uptake in several tissues. First data indicate that daily glucose uptake is highest before dark onset. This matches the rhythm of insulin sensitivity. Besides insulin, the glucogenic hormone glucagon is important in glucose metabolism. We found a daily plasma glucagon rhythm that is modulated by the SCN and by feeding³. However, neither of the aforementioned rhythms is likely to be causative to the glucose rhythm. Glucose and free fatty acids (FFA) compete for oxidation and thus, a possible FFA rhythm may interact with daily glucose homeostasis. Another question is therefore whether such a rhythm in lipid metabolism exists. First results indeed show a daily rhythm in plasma lipids. Its impact on the glucose rhythm remains unclear so far. In conclusion, the SCN modulates daily glucose homeostasis in many ways. However, for daily basal glucose levels, neural control seems to be more important than hormonal control.

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[DOI:10.1240/sav_gbm_2003_m_000362]

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Functional elements in the *Neurospora* circadian clock protein FREQUENCY

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FREQUENCY (FRQ), White Collar 1 (WC-1) and White Collar 2 (WC-2) are central components of the circadian clock in *Neurospora crassa*. WC-1 and WC-2 form a complex (WCC) that drives expression of *frq* RNA1 (1-3). In the course of a circadian day FRQ is progressively phosphorylated and degraded. Two PEST elements in FRQ, PEST-1 and PEST-2, are phosphorylated in vitro by recombinant casein kinase CK-1a (4). Single or double deletions of the PEST sequences result in hypophosphorylation of FRQ protein in vivo and arrhythmic condiation of the mutant strains. As shown previously, deletion of PEST-1 leads to a reduced rate of FRQ protein turnover (4). Deletion of PEST-2 does not affect the rate of FRQ turnover. We show that two serine residues in the PEST-2 are phosphorylated by CK-1a in vitro. Ser to Asn substitution of these residues results in an arrhythmic condiation phenotype. The mutant strain, *frqS4.5N*, expresses normal levels of the mutant FRQ protein but reduced levels of WC-1. However, levels of *wc-1* RNA are not reduced. Overexpression of WC-1 under control of the inducible *qa*-promoter restores overt rhythmicity of *frqS4.5N*. The data suggest that phosphorylation of the PEST regions in FRQ has specific and distinct functions in the circadian feedback loop. Phosphorylation of PEST-1 regulates FRQ protein turnover and phosphorylation of PEST-2 promotes expression of WC-1 in a post-transcriptional manner.

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[DOI:10.1240/sav_gbm_2003_m_000325]

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Extracellular long-term recordings of the accessory medulla, the circadian pacemaker of the cockroach *Leucophaea maderae*
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The temporal organization of physiological and behavioral states is controlled by circadian clocks in apparently all eukaryotic organisms. In the cockroach *Leucophaea maderae* transplantation studies (Reischig and Stengl 2003, J. Exp. Biol. submitted) located the circadian pacemaker in the accessory medulla (AMe). The AMe is densely innervated by peptidergic neurons, among them the pigment-dispersing hormone-immunoreactive (PDH-ir) neurons, some of which are also FMRFamide-ir. PDH is known to shift the phase of the locomotor activity at the late subjective day, when injected into the vicinity of the AMe in *L. maderae* (Petri and Stengl 1997, J Neurosci 17:4097-4093). We wanted to know whether PDH as well as the colocalized FMRFamide-like peptide phase delays the circadian pacemaker via suppression of action potential activity in pacemaker cells. In extracellular recordings of isolated AMe we tested in vitro whether AMe-neurons generate circadian rhythms in their action potential frequency and whether PDH- and FMRFamide peptides affect their electrical activity. We excised AMe with associated PDH-ir neurons with sharp glass pipettes and recorded from the explants with suction electrodes (0.3-2 MΩ). Recordings were performed under constant darkness, peptide injections were performed at specific Zeitgeber times with respect to the light dark cycles of the animal cultures. Tetrodotoxin (TTX)-blockable electrical activity was recorded for up to 5 days. We usually obtained multiunit recordings with several amplitudes ranging from 30 - 90 μV, but occasionally we also obtained single unit recordings of 110 μV. The maxima of event-frequency occurred at different Zeitgeber times for different units, but often preceded light-on or light-off. The activity oscillated with periods ranging from 6 h - 23.3 h. Thus, AMe neurons show oscillating electrical activity with different periods and varying phase relationships. To test whether PDH- and FMRFamide-like peptides affect the electrical activity of circadian pacemaker candidates we applied 150 fM of peptides with a micro injector to the excised AMe. In preliminary recordings we observed rises as well as decreases in electrical activity after application of PDH to different AMe cells at the late day. So far, we only observed increases in electrical activity after application of 100 fM FMRFamide at the middle of the day. We currently test whether peptide actions are restricted to specific Zeitgeber times and to specific physiological types of AMe neurons and whether the peptide effects can be simulated by cyclic nucleotides. [Supported by DFG grants STE531/7-3 and Human Science Frontiers]

[DOI:10.1240/sav_gbm_2003_m_000360]

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Norepinephrine-dependent phosphorylation of the transcription factor CREB in bovine pinealocytes

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In the rodent pineal gland, norepinephrine (NE)-dependent phosphorylation of cAMP response element-binding protein (CREB) leads to transcriptional activation of the melatonin rhythm-generating enzyme, arylalkylamine N-acetyltransferase (AANAT), and to increased expression of the clock gene *Per1*. In ungulates, however, NE appears to regulate melatonin production via posttranslational mechanisms, and it is not yet known whether transcription factors play any role in ungulate pineal functions. By use of a phosphorylated (p) CREB-specific antibody we show that NE induces a strong immunosignal in the nuclei of cultivated cells that were isolated from the bovine pineal gland and identified as pinealocytes by immunocytochemical demonstration of the melatonin precursor serotonin. Immunoblots reveal that this signal was caused by phosphorylation of CREB and another protein, presumably the transcription factor ATF-1. NE also increased AANAT protein; however, this increase did not seem to depend on transcriptional activation of AANAT because (i) the NE-induced rise in AANAT protein was also observed in the presence of actinomycin and (ii) AANAT mRNA levels did not change upon NE stimulation. Our results indicate that NE elevates pCREB levels in the pineal gland of all mammalian species investigated so far, irrespective of whether melatonin production is controlled via transcriptional mechanisms or not. Apparently, NE-induced CREB phosphorylation represents a very conserved element in pineal physiology of mammals. However, the genes targeted by pCREB may vary among different species. It remains to be shown whether, in the bovine pinealocyte, CREB phosphorylation leads to increased clock gene expression, as is true for the rodent pinealocyte.

[DOI:10.1240/sav_gbm_2003_m_000342]

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Behavioral phase shifts in mice with circadian clock gene deletions

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In the dual oscillator hypothesis by Daan et al (2001) the *cry1* and *per1* gene are associated with a molecular mechanism driving the Morning (M) component of the circadian oscillator and the *cry2* and *per2* gene are associated with the Evening (E) component. The shortened intrinsic circadian period length in mice deficient for one of the M genes and the extended period length in mice deficient for one of the E genes support this hypothesis. In addition, the circadian system in mice with a deficiency in one of the M genes should be able to show phase delays but no advances. On the contrary, the circadian pacemaker in mice knockout for one of the E genes should be able to show phase advances but no delays. Here we present phase shifts in *cry1*, *cry2*, *per1*, *per2* and wildtype mice evoked by exposure to a 15 min. (480 Lux) light pulse, both in freerun in DD and just after entrainment. This study supported by the EC Braintime project.

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[DOI:10.1240/sav_gbm_2003_m_000290]

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N-Methyl-Norsalsolinol perturbs normal diurnal rhythm in freely moving rats.

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The TIQ derivative N-methyl-norsalsolinol (NMNorsal) was identified as endogenously synthesized neurotoxin in parkinsonian lumbar cerebrospinal fluid. However, in former studies we could demonstrate that intraperitoneal injected NMNorsal is also able to pass through the blood-brain barrier of the rat. In vivo microdialysis in conscious, freely-moving rats were applied to examine the effect of intraperitoneally injected NMNorsal (20 mg/kg resp. 40 mg/kg). All in vivo experiments were performed between 10:00 and 14:00 hours and the behavioural activity of the animals was assessed during 48 h after NMNorsal injection. Behavioural activity was classified in arbitrary units for each animal. Additionally, levels of serotonin (5-HT) were measured in the caudate nucleus with microdialysis 1 h, 24 h, and 48 h following NMNorsal administration. During the experiments, low-dose-treated rats were almost always sleeping, but high-dose-treated (40 mg/kg) rats were active with a significant increase of mean behavioural score 24 hours (9 points) and 48 hours (15.6 points) after NMNorsal injection when compared to controls (1.3 points). It is noteworthy that the behavioural activity was clearly different to the classical 5-HT behavioural syndrome. After high-dose NMNorsal application, 5-HT levels increased to approximately 2-fold during the 48 hours while HIAA decreased to approximately 50 % in the dialysate. Since striatal 5-HT is released from neurons of the dorsal raphe nuclei and these nuclei are responsible for day/night-cycle in rats we suppose a linkage between increased behavioural activity of the animals, increased 5-HT levels in the caudate nucleus and NMNorsal application. Furthermore, the present experimental procedure could be useful as an animal model to study the context of 5-HT metabolism and sleep disturbance with regard to Parkinson's disease.

[DOI:10.1240/sav_gbm_2003_m_000334]

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Phase-dependent response of the clock protein KaiC to the dark pulse in cyanobacteria

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A circadian clock can be reset by a several-hour dark pulse in the cyanobacterium *Synechococcus elongatus* PCC 7942. KaiC is a master regulator to function in the negative feedback loop of the circadian clock, and temporal and transient overexpression of KaiC can reset the clock in *Synechococcus* (1). The KaiC accumulation and phosphorylation levels show circadian cycling peaking at early subjective night (CT 12-16) (2). To investigate the mechanism of the phase shifting, the effects of dark pulses on circadian accumulation and phosphorylation of KaiC were examined. In the subjective day (CT 5-16), KaiC accumulation and phosphorylation levels increase. A 5-h dark pulse beginning from CT 5 caused the largest phase delay in the *kaiBC* expression rhythm. During the dark treatment at CT 5-10, KaiC was accumulated and phosphorylated as did a control without the dark pulse. After being released from the dark pulse to constant light condition, the amount of KaiC was rapidly reduced. A dark pulse at CT 7.5-12.5 caused the largest phase advance. The effects of the dark pulse on KaiC accumulation and phosphorylation at CT 7.5-12.5 were different from those at CT 5-10. The dark pulse at CT 7.5-12.5 prohibited an increase in the KaiC accumulation level. Moreover, the ratio of phosphorylated KaiC elevated with the dark pulse more than in the untreated control strain. After the dark pulse, the amount of KaiC was gradually reduced. A possible connection between these effects on KaiC accumulation/phosphorylation and the phase shifting of the clock will be discussed.

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[DOI:10.1240/sav_gbm_2003_m_000385]

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Photoperiod differentially regulates clock genes expression in the suprachiasmatic nucleus of Syrian hamster

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The suprachiasmatic nuclei (SCN) contain the master circadian pacemaker in mammals. Generation and maintenance of circadian oscillations involve clock genes which interact to form transcriptional/translational loops and constitute the molecular basis of the clock. There is some evidence that the SCN-clock can integrate variations in day-length, i.e. photoperiod. However, the effects of photoperiod on clock gene expression remain largely unknown. We here report the expression pattern of *Per1*, *Per2*, *Per3*, *Cry1*, *Cry2*, *Bmal1* and *Clock* genes in the SCN of Syrian hamsters when kept under long (LP) and short (SP) photoperiod. Our data show that photoperiod differentially affects the expression of all clock genes. Among the components of the negative limb of the feedback loop, *Per1*, *Per2*, *Per3*, *Cry2* but not *Cry1* genes show a shortened duration of their peak expression under SP compared to LP. Moreover, mRNA expression of *Per1*, *Per3* and *Cry1* are phase advanced in SP compared to LP. *Per3* shows a mRNA peak of higher amplitude under SP conditions whereas *Per1* and *Per2* peak amplitudes are unaffected by photoperiod changes. *Bmal1* expression is phase advanced without a change of duration in SP compared to LP. Furthermore, the expression of *Clock* is rhythmic under SP whereas no rhythm is observed under LP. These results provide further evidence that the core clock mechanisms of the SCN integrate photoperiod.

[DOI:10.1240/sav_gbm_2003_m_000380]

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The hypothalamic suprachiasmatic nucleus cultured on multi-microelectrode arrays as a tool to study the circadian output from the biological clock

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The hypothalamic suprachiasmatic nucleus (SCN) is the primary circadian pacemaker (biological clock) in mammals that controls various rhythms of behaviour, metabolism and physiology. These rhythms are synchronized with the external light/dark cycle by retinal photoreception and transmission of light information via the retinohypothalamic tract. This information is integrated by SCN neurons and transmitted to different neuronal targets: endocrine neurons and autonomic neurons in the hypothalamus, as well as to areas outside of the hypothalamus. The role of the SCN in adjusting the activity of target neurons with the clock signal is not clear at present. In the present study we cultured hypothalamic brain slices that included the region of the SCN on multi-microelectrode arrays (MEA) to study in long-duration recordings simultaneously the electrical activity of SCN neurons and their possible target neurons in the hypothalamus. Extracellular recordings from acute hypothalamic slices and from organotypic slices mainly exhibited multi-unit activity, mostly without the possibility to discriminate single unit activity. The multi-unit activity was clearly, and reversibly, reduced or completely inhibited by the application of GABA (100 μ M) or by tetrodotoxin (TTX, 1 μ M). Neurons within the mouse SCN and within regions adjacent to the SCN, including the paraventricular nucleus of the hypothalamus (PVN), expressed circadian rhythms in spontaneous firing rate with periods of 23.5 to 24.5 hours. The circadian rhythm in hypothalamic areas outside of the SCN disappeared after removal of the nucleus showing that the rhythm is generated by SCN neurons. In acute slice preparations the mean activity showed a peak near midday, at CT 7.0 (CT = circadian time), whereas in organotypic slice cultures the time of peak activity was considerably shifted, due to the absence of a retinal input and the lack of a synchronizing stimulus that is able to adjust the rhythm. The time of peak activity was stable across several cycles in both preparations.

[DOI:10.1240/sav_gbm_2003_m_000331]

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Extra-retinal photoreceptors synchronise the circadian clock of *Drosophila melanogaster*.

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Synchronisation of the circadian clock in *Drosophila melanogaster* is achieved by light signals received through rhodopsins (Rh) and *cryptochrome* (CRY). Mutants blocking light signals from both compound eyes and CRY simultaneously affect synchronisation drastically, but do not make the circadian clock absolutely blind. This suggests the existence of additional extra-retinal photoreceptors. Also, molecular synchronisation of PERIOD and TIMELESS in "small ventral Lateral Neurons"(sLN_v)-behavioural pacemaker cells in the fly's brain- is still possible in these mutants, pointing to alternate mechanisms for light-dependent TIM degradation in addition to the known CRY-dependent mechanism. In order to investigate the H-B eyelet's role in circadian light reception we blocked the synaptic transmission from the H-B eyelet to the sLN_v by expressing tetanus toxin in this structure. We could demonstrate that the H-B eyelet indeed has a role in circadian photoreception, probably specialised to detect low intensity light. Additional candidates for extra-retinal photoreceptors are a group of clock-gene expressing cells in the dorsal brain, called the Dorsal Neurons (DNs). We generated a *period-luciferase* fusion gene, which is specifically expressed in the DNs. DN expression of this transgene is sufficient to mediate synchronised molecular oscillations and behaviour in the absence of functional eyes and CRY, together suggesting that they indeed might act as photoreceptors. Application of different photoreceptor mutants revealed that CRY is involved in light synchronisation of the DNs, but that other photopigments also contribute.

[DOI:10.1240/sav_gbm_2003_m_000354]

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Light does not degrade BMAL1 protein in the mouse SCN

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Biological rhythms in mammals are driven by a central circadian clock located in the suprachiasmatic nucleus (SCN). At the molecular level the biological clock is based on the rhythmic expression of clock genes. Two basic helix-loop-helix (bHLH)/PAS-containing transcription factors, CLOCK and BMAL1 (MOP3) provide the basic drive to the system by activating transcription of negative regulators, the mPer and mCry genes, through E box enhancer elements (Reppert & Weaver 2002). A critical feature of circadian timing is the ability of the clockwork to be entrained to the environmental light/dark cycle. The light-resetting mechanism of the mammalian circadian clock is poorly understood. Light-induced phase shifts are correlated with the induction of mPer1 and mPer2 and a subsequent increase in mPER1 protein levels (Field et al. 2000). It has been suggested that rapid degradation of BMAL1 protein in the rat SCN is part of the resetting mechanism of the central pacemaker (Tamaru et al. 2000). Using both immunocytochemistry and immunoblot, we found that BMAL1 and CLOCK proteins are continuously expressed in the mouse SCN, supporting the hypothesis that rhythmic negative feedback plays the major role in rhythm generation in the mammalian pacemaker. Moreover, we found that BMAL1 protein in the mouse SCN is not affected by a phase-resetting light pulse. These results indicate that rapid degradation of BMAL1 protein is not a consistent feature of resetting mechanisms in rodents. Supported by the DFG

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[DOI:10.1240/sav_gbm_2003_m_000317]

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Analysing Interrelations between Physiological and Molecular Events during Photoperiodic Flower Induction

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Physiological and molecular events during photoperiodic flower induction were compared in a short-day plant *Chenopodium rubrum* to fill in the gap between the knowledge on molecular regulation via transcription factors and their physiological action. *C. rubrum* has an absolute photoperiodic requirement for flower induction, and exactly characterised kinetics of the inductive process. From a network of transcription factors controlling the response of plants to different photoperiods and flower development, as analysed in detail in *Arabidopsis*, we choose *LEAFY*. We have identified a sequence for a LEAFY-like protein in *C. rubrum*, and analysed its expression in the course of flower induction using Northern blotting. We have analysed and quantified early physiological changes at the apical meristem in the course of flower induction, that involve changes in intracellular ion levels, shape of the apex and carbohydrate metabolism, as shown by intracellular fluorescent indicators and CLSM. Fluo 3 and Fura Red were used for calcium and carboxy SNARF-1 for pH (all Molecular Probes Europe, Leiden, The Netherlands). Changes of shape of the apical meristem were quantified using cryo REM. As all the changes point to the possible involvement of turgor changes at the apex in flower initiation, we have analysed expression kinetics of sucrose synthase, as a key enzyme in carbohydrate metabolism, and compared it with the physiological events and with the expression pattern of *LEAFY*.

[DOI:10.1240/sav_gbm_2003_m_000387]

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Molecular Control of Flower Initiation: Rhythms in Redox State as a Basis for Hydro- Electrochemical Signal Transduction

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With the short-day plant *Chenopodium rubrum* evidence is presented that a circadian rhythm (CR) in the network of energy metabolism could be the result of a compensatory control oscillation between rhythms in glycolysis, oxidative pentose-phosphate cycle, oxidative phosphorylation coupled to photophosphorylation in green cells (1, 2). The metabolic network gives rise to and most likely is controlled by endogenous rhythms in redox- and energy state. Photomodulation of the photoreceptor phytochrome leading to transient changes in pyridine nucleotide pool size levels (3, 4) might via changes in nucleotide ratios (e.g. NADP/NADPH) regulate transcription, translation and post-translational modulation. The recently observed redox regulation of plant homeodomain transcription factors (5) might thus be linked to photoperiod via photoreceptors like phytochrome and cryptochrome. The rhythmic integration of the plants is obvious from rhythmic changes in electric surface membrane potential. The symplastic organisation of higher plants is the basis for the hydraulic-bioelectric integration as displayed in rhythms in stem extension rate and leaf movements. Monitoring electric activity of plants we obtained electrophysiogrammes (EPGs) specifically correlating with photoperiodic flower induction. Taking into account the CR in photophile and scotophile phases we induced flowering under non-inducing photoperiods by phase-specific electrostimulation. Bioelectric responses are paralleled by turgor controlled rhythms in stem extension rate and leaf movements reflecting the hydro-electric communication and integration of the plant as a whole.

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[DOI:10.1240/sav_gbm_2003_m_000356]

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Pineal Clock Function in Wild Living Animals: Birds as Model Organisms

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The solar day has been the major and most consistent environmental factor influencing life since its origin. Hence, the temporal organisation of most organisms is characterised by a sequential occurrence of activity and rest. At the most basic level, rhythms are generated within cells which contain a particular molecular clockwork, but, to be effective for the organism, these molecular oscillations have to be transduced within the clock cell to change its activity and, in multicellular organisms, outside the cell to induce daily changes in behaviour or general physiology. Most of our knowledge on circadian clock function is derived from domestic inbred animals that are shielded from natural temporal variations of the environment. Due to their immense adaptive radiation and their enormous variety of life history strategies, birds are an exceptional model to study biological rhythms since it is obvious that their ecological diversity is somehow reflected in the organisation of their biological clocks, particularly the pineal gland of birds that plays a central role in their temporal organisation, both in a daily and in a seasonal context. We use a multi-methodological approach, including immunocytochemistry, microphysiometry, in vivo and in vitro melatonin measurements as well as molecular biology to investigate regulatory mechanism underlying the diversity and plasticity of pineal function in birds.

[DOI:10.1240/sav_gbm_2003_m_000322]

Functional Proteomics of Circadian Expressed Nucleic-Acid Binding Proteins in *Chlamydomonas reinhardtii*

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In *Chlamydomonas reinhardtii* several circadian rhythms are known including phototaxis, chemotaxis and cell division (Johnson et al., 1992). This green alga is ideally suited for proteomic analysis with prior purification steps, since it can be easily and quickly grown up in high amounts. Further, there are more than 180 000 EST's available and its entire genome is currently being sequenced. Our project aims to identify circadian expressed nucleic-acid binding proteins by applying functional proteomics. For this purpose, cells grown under an LD cycle were put under constant conditions of dim light and harvested at four different timepoints (LL25, LL29, LL37, LL41). Crude soluble protein extracts were prepared and nucleic-acid binding proteins were enriched by heparin-affinity chromatography. Heparin is well known for its binding affinity to nucleic-acid binding proteins (Jaques L.B., 1980). Interacting proteins were eluted with high salt, dialysed, acetone precipitated and separated by standardized two-dimensional gel electrophoresis. So far, we could identify three proteins, whose expression pattern changes during a circadian cycle. In future, these proteins will be in gel tryptic digested and the peptides will be identified via electrospray ionization mass spectrometry. Mass spectrometry data will be used to identify putative proteins from the available data bases.

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[DOI:10.1240/sav_gbm_2003_m_000363]

A novel EMS mutation demonstrating light-dependent interaction of TIM and CRY proteins *in vivo*

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A chemical mutagenesis screen was performed with flies carrying a *period-luciferase (per-luc)* construct, which has been demonstrated to reflect the rhythmical expression of *per*-RNA *in vivo*. 5137 lines were tested for an altered *luciferase* cycling in 12:12 hour LD condition. A novel EMS mutant that emerged from this screen maps to the second chromosome. A complementation assay with various *tim*-null alleles suggested that the new mutant represents a hypomorphic *tim*-allele. Sequence analysis revealed that the *tim* gene in the mutant encodes a protein with two closely located amino acid substitutions in the carboxyterminal part of the protein mapping to the *cryptochrome (cry)* interaction domain (Rosato et al., 2001). Western blot analysis showed that the light induced, phosphorylation dependent TIM protein degradation is decelerated in the early morning which point to a disturbed light entrainment. Therefore we named our mutant *tim^{blind}*. The period length of 25,9h in constant conditions in homozygous mutant flies, compared to 24h in wildtype flies identifies our mutant to be another period lengthening *tim*-allele. *tim^{blind}* flies nicely reentrain to delay and advance light regime shifts similar to *cry^b* mutant flies (Stanewsky et al., 1998). However in *tim^{blind}, cry^b* double mutant flies, reentrainment is strongly delayed for up to six days. This suggests - in agreement with our sequence data - a direct interaction of TIM and CRY proteins with each other and that this interaction is disturbed in the double mutant flies.

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[DOI:10.1240/sav_gbm_2003_m_000349]

Functional and neuroanatomical changes of the SCN: cause or consequence for spontaneous hypertension

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Recent human and animal experimental data have revealed the involvement of autonomic centers in hypothalamus in pathophysiology of hypertension. The number of VP containing neurons in SCN and CRH neurons in PVN are altered in human hypertensive brains (Ref.1.). Transplantation of hypothalamic tissue containing SCN and PVN from hypertensive animals to normotensive ones results in development of hypertension. Consistent with the anatomical data, hypertensive animals show several disturbances in their circadian rhythms (Ref.2.). Although, the considerable evidence exist on hypothalamic involvement of hypertension, most of the studies have focused on long term alterations. In the current study we aimed to investigate whether the observed hypothalamic changes are cause or consequence of hypertension. In order to evaluate changes in circadian physiology in the course of development of hypertension, a set of experiments is conducted with spontaneously hypertensive rats (SHRs) and their normotensive controls (WKYs), which are kept in circadian boxes between age of 4 and 16 weeks. During the experiments, both SHR and WKY are maintained in different light regimes to test the functionality of the biological clock before, during and after the development of hypertension and related anatomical changes will be evaluated with immunocytochemistry and *in situ* hybridization after every light session and different stages of hypertension.

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[DOI:10.1240/sav_gbm_2003_m_000341]

The relationship between melatonin and dopamine rhythms in the duck retina

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It has been suggested that in retinae of several vertebrates dopamine (DA) and melatonin (MEL) act as mutually inhibitory signals for light and dark, respectively, being involved in the regulation of several morphological and physiological processes that occur rhythmically and are under the influence of environmental lighting conditions [1-4]. In this study we investigated the relationship between DA and MEL in the duck retina. Concentrations of DA and its main metabolite, 3,4-dihydroxyphenylacetic acid (DOPAC), fluctuated throughout the 24 h period, with high values during the light phase. The rhythmic changes in DA content and metabolism in the duck retina were out of phase with the daily oscillations in MEL and serotonin N-acetyltransferase (AA-NAT; the penultimate and key regulatory enzyme in MEL biosynthesis) activity. Acute exposure of ducks to light at night potently increased levels of DA and DOPAC, and decreased AA-NAT activity and MEL content in the retina. Intraocular administration of MEL to light-adapted ducks produced a significant decline in retinal DA and DOPAC concentrations. On the other hand, quinpirole, a D2/D4-DA receptor agonist, administered intraocularly, markedly suppressed night-time retinal AA-NAT activity and MEL. These findings provide, for the first time, evidence for an inverse relationship between the DA system and MEL in the duck retina. Supported by the Wellcome Trust grant No. 064230/Z/01/Z to D.J. Skene and J.B. Zawilska.

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[DOI:10.1240/sav_gbm_2003_m_000388]

Models of metabolic feedback on circadian gene expression

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Circadian oscillations, of about 24 h are property for most organisms to anticipate daily changes in the environment.. (A.T. Winfree, 2000). In many organisms rhythm generation is attributed to negative feedback cycles of circadian clock gene expression, with the expressed clock proteins diffusing back to the nucleus in order to inhibit their own expression (J.C. Dunlap, 1999). Recently, a feedback from the state of metabolism onto circadian gene expression has been demonstrated (J.Ritter at al, 2001, Stokkan at al., 2001) In this work, metabolic feedback on circadian gene expression is studied with a minimal model describing mRNA and protein production and degradation by a system of two coupled nonlinear delay differential equations (Scheper at al., 1999). This system is coupled with second system which describes the protein, substrate and product reaction defined by two ordinary differential equations. The linear stability analysis shows that one the main property of circadian rhythms, robustness of oscillations, exists and that the period remains around 24 h for a wide range of parameters. Although the period remains the same, the amplitude of mRNA abundance is changing according to the changes of parameters. The metabolic influence is studied by means of few parameters, which couple both systems with each other. Using theoretical approach, it was found that metabolic activity could inhibit the gene expression in a certain range of parameters. The model also takes account presence of internal noise e.g., the stochastic nature of biochemical reactions described in this model.

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[DOI:10.1240/sav_gbm_2003_m_000391]