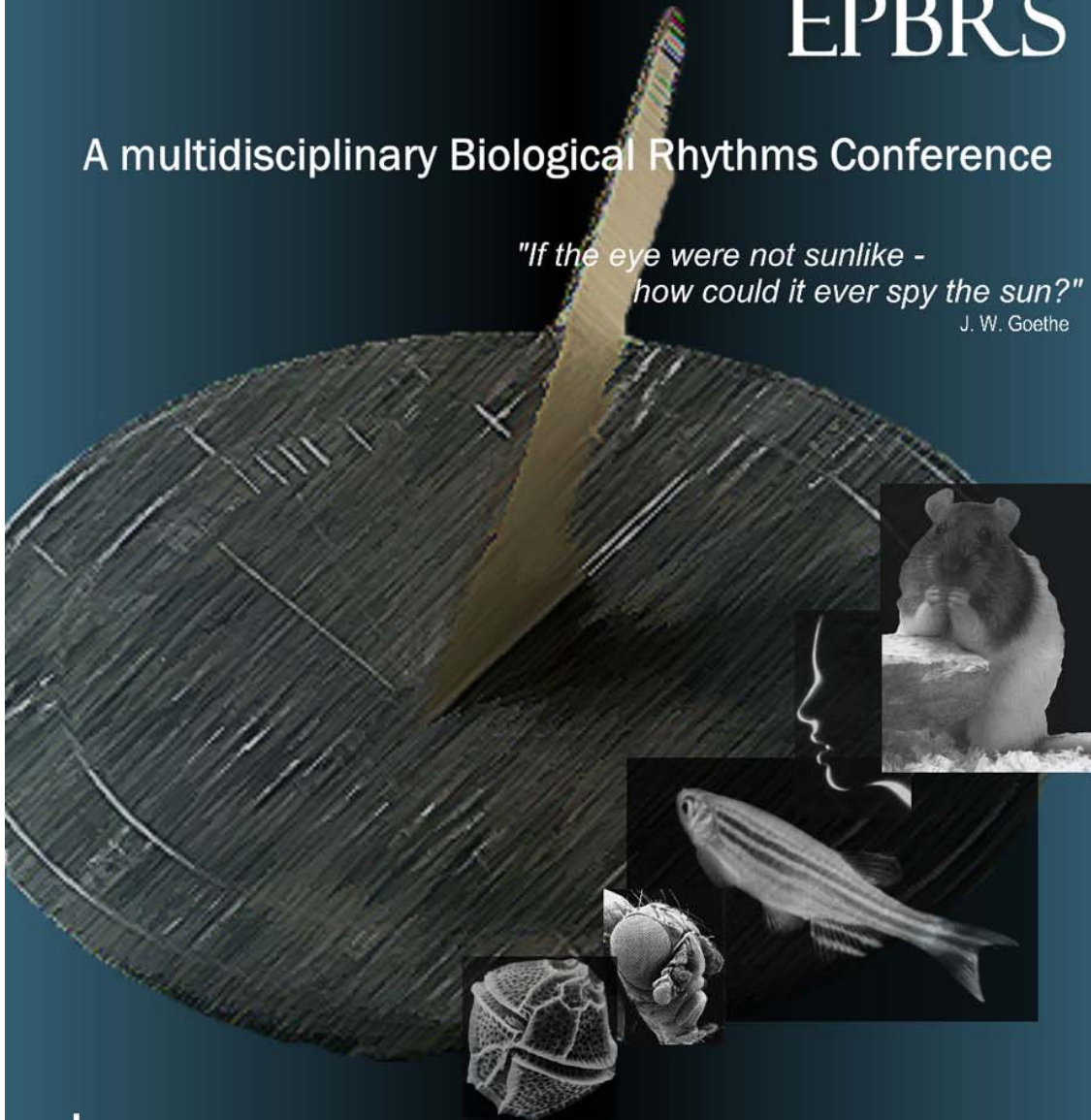


X. CONGRESS OF THE EPBRS

A multidisciplinary Biological Rhythms Conference

*"If the eye were not sunlike -
how could it ever spy the sun?"*

J. W. Goethe



epbrrs

J. W. Goethe-University Frankfurt/Main, September 1 - 5, 2005

Lectures and oral communications

(Arranged according to the time schedule)

Thursday, September 1, 2005

MELATONIN IN HUMANS

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The pineal hormone melatonin, via its secretion pattern during the hours of darkness, is the only solidly established humoral method of signalling time of day and time of year to other physiological systems. This function underpins its all-pervasive presence in chronobiology. Melatonin is also supremely important for research on the physiology and pathology of the human biological clock. Its profile in plasma or saliva provides the best available measure of circadian timing, of response to changing daylength and of non-image forming responses to ocular light. Its primary metabolite, 6-sulphatoxymelatonin in urine allows long term monitoring of human rhythms in 'real life' situations where rhythms may be disturbed. Melatonin is not only a 'hand of the clock': endogenous melatonin acts to reinforce the functioning of the human circadian system, probably in many ways. Most is known about its relationship to sleep and the decline in core body temperature at night. Current perspectives also include a possible influence on major disease risk, arising from circadian rhythm disruption. Treatment with melatonin induces sleepiness and lowers core body temperature during 'biological day' (when endogenous melatonin is low) and the timing (phase) of human rhythms can be changed when treatment is appropriately timed. Melatonin is able to entrain free running rhythms and maintain entrainment in most blind and some sighted people. Used therapeutically it has proved a successful treatment for circadian rhythm disorder, particularly the non-24h sleep wake disorder of the blind, delayed sleep phase syndrome and (less consistently) the problems of shift work and jet lag. Numerous other clinical applications are under investigation. However areas of controversy and large gaps in knowledge mean that general conclusions cannot be drawn with regard to most situations. The future holds much promise for melatonin as a research tool and as a therapeutic treatment.

MELATONIN AND THE CIRCADIAN CLOCK IN MAMMALS

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The temporal organization of living organisms relies on clocks that generate rhythms and that are capable of being entrained to environmental factors. Such clocks convey circadian information to the rest of the organism via nervous and/or endocrine pathways. Melatonin (Mel) secretion by the pineal gland during the night is under control of the main/master circadian clock within the suprachiasmatic nuclei (SCN). The Mel rhythm is thus an efferent hormonal signal from the clock which can be used as a circadian mediator to any structure that can "read" it. Moreover, the duration of the nocturnal Mel secretion, which is proportional to the length of the night, allows the brain to integrate the photoperiod. Thus, Mel appears to convey photic information that is used for temporal organization of circadian and seasonal rhythms. In mammals, through involvement of SCN Mel receptors, exogenous Mel can be used as a pharmacological tool to manipulate circadian processes (so-called "chronobiotic effect"). In rodents, Mel entrains free-running circadian rhythms but only when the administration time coincides with the light/dark transition (e.g., onset and offset of activity in nocturnal and diurnal mammals, respectively) and the phase angle difference depends on the duration of the Mel signal. We demonstrated also that Mel administration directly within the SCN (i.e., by microdialysis) deeply affects clock activities (long term increase in oscillation amplitude of some clock outputs). The molecular and physiological mechanisms underlying these effects of exogenous Mel are not clarified yet. Contrary to what has been described for photic and most non-photic cues, we observed that clock gene mRNAs are not the initial targets for Mel action in the suprachiasmatic nuclei. This suggests that Mel affects the clock either at a post-translational mechanism within the cellular machinery or at a physiological level (i.e., output pathways within the SCN). Our observation that Mel can inhibit the behavioural phase-shifting properties of serotonergic stimulation without affecting the effect of 8-OH-DPAT on *Per* mRNA levels in the SCN supports this hypothesis. Recent data on newly identified clock elements, however, do not permit to totally exclude a direct effect of Mel on the molecular machinery of the mammalian clockwork.

PACAP, A MULTIFACETTED NEUROPEPTIDE

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PACAP (pituitary adenylate cyclase activating polypeptide) was originally isolated from ovine hypothalamus based on its ability to stimulate cAMP production in pituitary cell cultures. The peptide exists in two forms which both are derived from the same precursor: PACAP38 and the C-terminally truncated PACAP27, which both can interact with three subtypes of receptors activating adenylate cyclase and/or phospholipase C. PACAP is considered to be a hypophysiotrophic factor, but its effect on pituitary hormone release is less dramatic than that of the classical releasing hormones. We have provided evidence that PACAP could be a hypothalamic hypophyseal regulatory factor during acute inflammatory stress. PACAP is not only expressed in the hypothalamus but widely distributed in the central and peripheral nervous system and in peripheral organs and has been shown to act as hormone, neurohormone, autocrine/paracrine substance, neurotransmitter, neuromodulator, neurotrophic factor and immunomodulator. Two examples on the functional role of PACAP in biological timing will be presented: 1) The transient expression of PACAP during the periovulatory period in ovarian cells in which PACAP functions as autocrine/paracrine inducer of progesterone secretion and subsequent luteinization in periovulatory granulosa/lutein cells, and 2) the functional role for PACAP as neurotransmitter or neuromodulator in regulation of the brain's biological clock

HYPOCRETINS: THE TIMING OF SLEEP AND WAKING

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It is an important advantage in Darwin's sense to be able to choose the right time and the right place for sleep and waking. The brain structures involved in the maintenance of wakefulness include cholinergic, monoaminergic, serotonergic and histaminergic projections to the thalamus, hypothalamus, basal forebrain and the cerebral cortex. Gabaergic neurons in the ventrolateral preoptic area on the other hand fire during slow wave sleep and inhibit the arousal systems. A fatal insomnia is associated with lesions of this area in the anterior hypothalamus. Lesions in the posterior hypothalamus – including the histaminergic and hypocretinergic nuclei - induce hypersomnia (encephalitis lethargica von Economo). The histaminergic neurons in the tuberomammillary nucleus display a pacemaker-like activity during waking and are inhibited during sleep, they project to the whole central nervous system and interact specifically with the hypocretin neurons and the suprachiasmatic nucleus. Hypocretin (orexin) - neurons in the perifornical area seem to play the role of a conductor in the aminergic orchestra. They have been suggested to provide a flip-flop switch that resists rapid and frequent transitions between sleep and waking (Saper et al., 2001). These neurons were identified by two groups in 1998 (DeLecea et al. and Sakurai et al.) and first thought to be involved in the regulation of food intake. Their degeneration, probably as a result of an autoimmune attack, causes narcolepsy, a debilitating inability to separate sleep and waking; the loss of muscle tone normally experienced during REM sleep occurs sudden out of the waking state (cataplexy). Day time sleepiness is matched with disrupted night sleep and hypnagogic hallucinations during sleep paralysis. Orexin neurons are excitatory to the arousal systems, but they target thalamus, hippocampus and cerebral cortex, indicating an even larger integrative role.

THE EVOLUTION OF MELATONIN GENERATING SYSTEMS

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Melatonin rhythm generating systems generate the daily rhythm in melatonin found in all vertebrates. At the heart of these systems is the pineal gland – the melatonin factory - which has the ability to convert high amounts of tryptophan to melatonin. Two other elements complete these systems: one is an endogenous ~24 hour clock; the other is a photodetector which resets the clock and controls clock output. All three functions, melatonin production, 24 hour timing and light detection are found in the pineal cells of fish, birds and other submammals. The mammalian melatonin rhythm generating system is decentralized: the clock is the master 24- hour oscillator in the suprachiasmatic nucleus in the hypothalamus; and, light is detected in the retina. A neural pathway connects these elements and the pineal gland. It should be added that some of these features are also found in cells in vertebrate retinæ. However, the retinal production of melatonin is severely limited relative to that in the pineal gland; moreover, in primates essential elements required for melatonin synthesis are absent from the retina. Melatonin rhythm generating systems have a unique role in physiology, which is to convert night and day into the circulating melatonin signal that is used to tell time. This provides a means to control daily rhythms in metabolism and activity, thereby optimally integrating these functions with the environment. High levels of melatonin signal night. Environmental lighting acts through melatonin rhythm generating systems to organize and coordinate the complex daily rhythms of vertebrates within diverse ecological systems. How did melatonin rhythm generating systems evolve? I have proposed that the dominant pressure that initiated this evolutionary vector was the toxic nature of some chemicals in the environment of our primitive vertebrate ancestors. The specific chemicals are arylalkylamines, a broad class of chemicals characterized by an aromatic (aryl) group with a carbon side chain (alkyl) terminating in a primary amine. Amines are potentially toxic, because they are highly reactive by virtue of conversion to a reactive aldehyde or through their reaction with aldehydes to form Schiff bases. This reactivity is seen as having the greatest impact on survivability in the context of the photoreceptor, where photodetection is a direct reflection of the abundance of an essential photochemical, retinaldehyde. Retinaldehyde is limited in availability. Capture of each photon of light leads to the recycling of retinaldehyde from an inactive trans- form to an active cis- form. The hypothesis of pineal evolution proposes that melatonin production started as an enzymatic mechanism to prevent arylalkylamines from depleting retinaldehyde, specifically by acetylating arylalkylamines. This reflected the acquisition of arylalkylamine *N*-acetyltransferase, which is the next to last step in melatonin synthesis. Introduction of this enzyme into the photoreceptor of a primitive vertebrate ancestor was an advantage because it enhanced photodetection by preventing depletion of retinaldehyde. During the course of vertebrate evolution, descendants of this cell evolved in two directions, one leading to the melatonin producing pineal cell and the other to photosensitive retinal photoreceptor cell. This theory unifies thinking about the evolution of melatonin rhythm generating systems and is of special interest because elements of it can be experimentally tested. Representatives of our early ancestral vertebrates which lack arylalkylamine *N*-acetyltransferase – the urochordates – can be used to determine the impact of this enzyme on photodetection.

Friday, September 2, 2005

THE CLOCK IN THE GENES: CLOCK PROPERTIES AND MECHANISMS IN CELLS

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Neurospora crassa has been one of the leading model systems for identifying clock genes and how they function within a transcription/translation negative feedback loop to control free-running rhythms. Circadian clocks in nature are, however, typically entrained, and we know far too little about the mechanisms that control this most relevant function of the clock in *Neurospora*. We are systematically using this simple cellular system to decipher these mechanisms, working from the physiological to the molecular level:

Despite apparent drivenness in symmetrical, non-24 h cycles (T-cycles), *Neurospora* shows systematic entrainment in photoperiods.

A 'circadian surface' was generated, covering entrainment conditions in 'all photoperiods' in different T's and in different clock mutant strains. The results reveal rules for phase relative to free running period and photoperiod.

T-cycles using temperature rather than light, involving clock mutant strains, show masking behaviour that can be distinguished from underlying circadian entrainment.

Temperature cycles using different thermoperiods (T=24 h) also show systematic entrainment.

In temperature cycle, clock gene RNA is regulated by the clock, rather than as a driven response to a zeitgeber.

In light cycles, clock gene RNA is driven by lights on and off signals, but clock protein is regulated by the clock.

Thus, principles of entrainment can be studied in the relatively simple system. The characteristics discovered so far reveal that control mechanisms of the *Neurospora* clock genes and their products are distinct in constant and entraining conditions.

DISRUPTION OF CRYPTOCHROME RESCUES CIRCADIAN RHYTHMICITY IN PERIOD-NULL DROSOPHILA MUTANTS

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In *Drosophila*, circadian rhythms are reflected in among other things, the daily locomotor activity cycles, both in DD, and in LD. In LD, flies show a characteristic anticipation of the “lights on” and “lights off” signals by increasing their locomotion. However, this is lost in period-null mutants. We have discovered that this locomotor anticipation of the light dark transition is restored when the per-null mutation is placed in combination with cryb, a severe mutation of cryptochrome, the dedicated circadian photoreceptor. This rescue of rhythmicity appears to be due to a residual oscillation that can be expressed when the light input to the clock is attenuated. We shall discuss this novel result in the context of the differential functions of the clock-expressing neurons of the fly.

CLOCK GENES IN MAMMALIAN CELL CLOCKS

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The cellular circadian core oscillator is thought to be composed of an autoregulatory transcription-translation feedback loop involving a set of clock genes, where a negative loop plays a dominant role. In fibroblasts where *mPer2* expression is controlled through tetracycline-regulatable promoter, we revealed that constitutive and over-expression of *mPer2* mRNA severely impairs serum shock induced cyclic circadian clock gene expressions. Even in constitutive *mPer2* mRNA expression, mPER2 protein accumulation in these cells showed clear circadian oscillation, suggesting that the protein cycling of mPER2 was required for oscillation of the circadian feedback loop. Although genetic and molecular evidences confirm *mPer2* as the main oscillator gene in mammals, the role of *mPer1* is still unclear. By the prolongation of the lighting period, we revealed a large delay-phase shift of behavioral rhythm in *mPer1*-deficient mice with the loss of PER2 at protein level. This suggests that *mPer1* acts as a circadian adaptor gene adjusting central oscillator *mPer2*. In invertebrates and vertebrates other than mammals, light signals directly reach peripheral cells and entrain local cell clocks via intrinsic photoreceptors. In mammals, however, light perception is restricted to the eye, and the retinohypothalamic tract conveys retinal light information to the circadian center in the suprachiasmatic nuclei (SCN) and entrains the clock. We recently revealed that external light stimuli induce profound changes of gene expression in the adrenal gland with the massive release of corticosterone. The magnitude of induction is in proportion to the light strength, suggesting that environmental light signals are instantly converted to glucocorticoid signals, which may possibly act as metabolic phase-resetter in peripheral cell clocks. Thus, the mammalian multidimensional circadian time conducting system also conveys environmental light information to cells in the whole body.

STRUCTURE - FUNCTION ANALYSIS OF MAMMALIAN CRY1

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Circadian clock-controlled rhythms provide organisms with an orchestrated temporal program that allows for appropriate timing of physiology and behavior. The mammalian circadian pacemaker is composed of a set of “clock” genes acting in autoregulatory transcription-(post)translation based feedback loops. The mammalian CRY1 and CRY2 proteins are central components in the negative limb of this circuit as they strongly inhibit CLOCK/BMAL1-mediated transcription. Mammalian CRY proteins belong to the photolyase/cryptochrome family of flavoproteins and were initially identified as homologs of the DNA repair protein photolyase, an enzyme that removes ultraviolet light induced DNA damage using visible light as an energy source. Although animal cryptochromes share a high degree of homology with photolyases, they lack the NLS containing N-terminal extension characteristic for eukaryotic photolyases, and instead contain a C-terminal extension as also observed in plant cryptochromes. By analysing a set of mutant mCRY1 proteins in various clock cellular assays, we found that the C-terminus can competitively interact with mPER proteins and BMAL1 and that it is involved in nuclear localization of the protein (through two separate pathways) and in transcription inhibition. In addition, sequences in the core domain (the 500 amino acid region homologous to photolyase) are required for CLOCK/BMAL1 inhibition through association with CLOCK. Apparently, the divergent evolution of the tail of mCRY1 (and likely mCRY2), distinguishing the protein from *Drosophila* and plant CRY proteins, has played an important role in establishing the mammalian clock circuit.

ROLES OF SERINE PHOSPHORYLATION IN REGULATION OF MOUSE CRYPTOCHROMES

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Post-translational modifications such as phosphorylation of circadian clock proteins appear crucial for the time-keeping mechanism. The clock proteins including CLOCK, BMAL1, PER1 and PER2 undergo temporal change in phosphorylation in the mouse liver, a well-characterized peripheral clock tissue, but it was unclear whether CRYs are phosphorylated *in vivo*. We found that CRY1 and CRY2 are phosphorylated by MAP kinase *in vitro*, and mass spectrometry analysis identified Ser265 and Ser557 of CRY2 as *in vitro* phospho-acceptor residues. Similarly, MAP kinase phosphorylated CRY1 at Ser247, a site corresponding to Ser265 of CRY2. An effect of the Ser phosphorylation was investigated by mutating Ser247 of CRY1 and Ser265 of CRY2 to Asp, which resulted in attenuation of each CRY's ability to inhibit BMAL1:CLOCK-mediated transcription. On the other hand, we found Ser557-phosphorylated CRY2 that accumulated in the liver during the (subjective) night in parallel with CRY2 protein, and the phosphorylated form reached its maximal level at late night preceding the peak-time of the protein abundance by approximately 4 hrs in LD and DD conditions. The Ser557-phosphorylated form of CRY2 was localized in the nucleus, whereas CRY2 protein was located in both the cytoplasm and nucleus. Functionally, phosphorylation of CRY2 at Ser557 allowed subsequent phosphorylation of the protein, resulting in efficient degradation of CRY2 by a proteasome pathway. The present study demonstrates an important role of priming phosphorylation at Ser557 for destabilization of mCRY2, and illustrates a model that the circadian regulation of CRY2 phosphorylation contributes to rhythmic degradation of CRY2 protein.

MOLECULAR MECHANISM OF TEMPERATURE-SENSING BY THE CIRCADIAN CLOCK OF *NEUROSPORA CRASSA*

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FREQUENCY (FRQ) is a central component of the circadian clock in *Neurospora*. *Frq* RNA is rhythmically expressed and synthesized with a 1519 nt 5'-UTR containing 6 upstream open reading frames (uORFs) and multiple introns. A large (l) and a small (s) isoform of FRQ are translated from a common primary transcript corresponding to translation initiation at AUG1 (Met1) and AUG3 (Met100), respectively. Expression levels and ratios of the long (l) and short (s) isoforms of FRQ are crucial for temperature compensation of circadian rhythms. We show that the ratio of l-FRQ vs. s-FRQ is regulated by thermosensitive splicing of intron 6 of *Frq*, a process removing the translation initiation site of l-FRQ. Thermosensitivity is due to inefficient recognition of non-consensus splice sites at elevated temperature. Temperature dependent accumulation of FRQ relative to bulk protein is controlled on the level of translation. The uORFs in the 5'-UTR of *Frq* RNA are in non-consensus context for translation initiation. Thermosensitive trapping of scanning ribosomes at the uORFs leads to reduced translation of the main ORF and allows adjustment of FRQ levels according to ambient temperature.

TEMPORAL AND SPATIAL EXPRESSION PATTERNS OF PHOSPHORYLATED FORMS OF ERK1,2 IN THE RAT SUPRACHIASMATIC NUCLEUS

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The suprachiasmatic nucleus (SCN) of the hypothalamus is the primary circadian clock in mammals. The molecular mechanism allowing the SCN to generate a stable circadian period consists of interlocking transcriptional/translational feedback/forward loops of clock genes and their protein products. Post-translational events such as the phosphorylation of clock proteins also contribute largely to the circadian mechanism. Phosphorylation of the extracellular signal-related kinase (ERK) 1/2 has been shown to exhibit a nycthemeral and circadian rhythm in a number of clock structures. Here we report a spatial and temporal regulation of p-ERK in the rat SCN. ERK activity was monitored after fluorescence labeling of SCN sections with an antibody against the phosphorylated forms of ERK, by densitometric analysis of confocal images. Immunostaining was greater in the dorsomedial part of the SCN during the day (ZT6) and in the ventrolateral part during the night (ZT18), revealing shell-like and core-like patterns respectively. To further characterize the neurochemical phenotype of the p-ERK-expressing cells, we used dual-label immunofluorescence for p-ERK and either AVP, GFAP, VIP or GRP. Irrespective of the time of day, there was no overlap between p-ERK and GFAP labelings. In contrast, at ZT6, a few cells were immunopositive for both p-ERK and AVP in the dorsal part and, at ZT18, good proportions of the p-ERK-positive cells clearly colocalized VIP or GRP. Then, although the rat SCN exhibits two rhythmic regions of p-ERK expression that are reminiscent of known SCN neurochemical subdivisions, the present data point to only partial overlap between p-ERK and AVP at daytime and between p-ERK and VIP or GRP at nighttime.

GASTRIN-RELEASING PEPTIDE RESTORES RHYTHMS TO THE SUPRACHIASMATIC CIRCADIAN CLOCK OF MICE LACKING THE VPAC2 RECEPTOR

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Vasoactive intestinal polypeptide (VIP) acting through the VPAC2 receptor is a key signaling pathway involved in synchronizing cellular activity of the suprachiasmatic nuclei (SCN) circadian clock to environmental lighting conditions and in pacemaker function itself. Thus, mice lacking the VPAC2 receptor (*Vipr2*^{-/-}) exhibit abnormal responses to light and impaired behavioral rhythms. However, it is still unclear whether individual neurons in the adult *Vipr2*^{-/-} SCN sustain circadian firing patterns. Further, it is now known that a minority of adult *Vipr2*^{-/-} mice express coherent circadian wheel-running rhythms but it is not known whether the SCN oscillate in these mice. Here we developed a novel suction electrode recording method to show that in behaviorally arrhythmic adult *Vipr2*^{-/-} animals the electrical activities of SCN neurons rarely show circadian variation. By contrast, in *Vipr2*^{-/-} mice exhibiting behavioral rhythms, the majority of SCN neurons express low amplitude electrical oscillations with periods matching those of the animals' behavioral activity. Remarkably, exogenous gastrin-releasing peptide (GRP), an SCN neuropeptide not normally required for circadian rhythms, restored SCN electrical oscillations to behaviorally arrhythmic *Vipr2*^{-/-} mice. Further, a GRP receptor antagonist blocked SCN neural rhythms in brain slices from behaviorally rhythmic *Vipr2*^{-/-} mice. Our results establish that signaling via the VPAC2 receptor is not essential for circadian rhythms, either in individual neurons or animal behaviors. Further, our data highlight a potential, and unexpected, new role for GRP in the regulation of SCN pacemaker activity. These findings therefore confirm the requirement for intercellular signaling in maintaining SCN clock function at the tissue level.

Supported by the BBSRC UK

FLUORESCENCE IMAGING OF THE SUPRACHIASMATIC CIRCADIAN CLOCKWORK: DEPENDENCE ON PEPTIDERGIC INTER-NEURONAL SIGNALLING

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Real-time video microscopy of long-term (10- 50 day in vitro) organotypic SCN cultures from *mPer1::dsGFP* mice revealed a robust, high amplitude cycle of circadian gene expression that was sustained over the course of weeks. Individual oscillatory regions of interest (ROI) that likely corresponded to individual neurons were in phase across the culture. High amplitude oscillation derived from the dorsomedial SCN, but the neurochemical identity of oscillatory ROIs was heterogeneous. Rhythmic gene expression was evident in areas devoid of VIP cells and typically mapped to AVP containing areas, with or without VIP cells. Other areas containing VIP cells (ventral core) or AVP cells (lateral shell) had very low amplitude cycles. Application of TTX acutely and reversibly dampened circadian gene expression, indicating that the core molecular clockwork is dependent on inter-neuronal signalling. In slices from mice lacking the VPAC2 receptor for VIP, no circadian gene expression could be detected. On initial preparation of the slices, however, strong *mPer::dsGFP* signal was acutely induced in the dorsomedial SCN, indicating that lack of VIP-mediated signalling does not affect acute regulation of *mPer1::dsGFP*, but it does compromise its circadian oscillation. We conclude that the core molecular loop of the SCN clock neuron, as reported by *mPer1::dsGFP*, is sustained by inter-neuronal signalling and that neuropeptidergic communication across the SCN assemblage mediated by the VPAC2 receptor is critical to maintain the cellular clockwork. These results overturn a simple “top-down”, linear view of the oscillatory SCN neuron in which the molecular timekeeper unilaterally determines membrane function, and emphasise the importance of neural circuitry in sustaining the molecular time-keeper.

PRESERVATION OF FUNCTIONAL CENTRAL RHYTHMICITY AND LIGHT ENTRAINMENT, BUT NOT PERIPHERAL RHYTHMICITY IN CLOCK MUTANTS

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The circadian rhythmicity of hormone secretion and sleep/wakefulness is the result of an endogenous rhythm in the suprachiasmatic nucleus (SCN) and entrainment by light via retinal inputs. The *Clock* gene has been considered essential for the generation of endogenous cellular rhythmicity both centrally and in the periphery. However we have shown previously that melatonin proficient *Clock* mutant mice (*Clock* + MEL) retain melatonin rhythmicity in LD and for at least 2 days in DD, suggesting central rhythmicity is intact. Here we show that under LD conditions expression of *Per2* and *prokineticin 2* mRNA in the SCN of *Clock* + MEL mice was rhythmic and that *npas2* mRNA was highly and constitutively expressed. Furthermore, melatonin production and wheel running activity in the mutants were rhythmic in 50% of mice after 14 days in constant darkness, with the remainder becoming arrhythmic. The wheel running rhythm could be entrained by single daily 15 minute 200 lux light pulses in DD in 40% of mice while a further 44% free ran. In those mice that entrained behaviourally, the melatonin rhythm was entrained. In contrast to the SCN, liver and skeletal muscle gene expression was arrhythmic in *Clock* + MEL mice in LD. *Bmal1*, *Per2*, *npas2*, *phosphofructokinase*, *Pepck* and *glucokinase* mRNA in the liver and *Bmal1* and *Per2* mRNA in muscle were arrhythmic in contrast to wild type mice. Plasma corticosterone secretion was also arrhythmic in the mutants. The *Clock* mutation does not appear to abolish central rhythmicity and light entrainment, suggesting that a functional *Clock* homologue exists in the SCN. Nevertheless, the SCN cannot maintain peripheral rhythmicity through rhythmic outputs including melatonin secretion in the absence of *Clock* expression in peripheral tissue. Therefore peripheral, but not central rhythmicity has an absolute requirement for *Clock*, with as yet unknown *Clock* independent factors able to generate the latter.

A NOVEL OCULAR PHOTORECEPTOR: EMERGING ROLES AND PHOTSENSORY MECHANISMS

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In the early 1990's studies on mice with hereditary retinal disorders, and lacking most of their rod and cone photoreceptors, were shown to adjust (entrain) their circadian rhythms to the environmental light/dark cycle with normal sensitivities. Collectively these experiments suggested that there might be another class of photoreceptor within the eye, but could not preclude the possibility that only small numbers of rods and/or cones were required for photoentrainment. Thus mice (*rd/rd cl*) were engineered in which all the rods and cones were functionally ablated. Circadian entrainment, and a variety of other responses to environmental irradiance (e.g. pupil constriction), were preserved in rodless+coneless mice and so demonstrated unambiguously the existence of a non-rod, non-cone ocular photoreceptor. The cellular localisation of the non-rod, non-cone ocular photoreceptors in mice utilised the isolated *rd/rd cl* retina in combination with calcium (Ca^{2+}) imaging techniques. This approach showed that the murine retina contains a plexus of electrically coupled directly photosensitive ganglion cells (dpRGCs) and that Ca^{2+} is likely to play an important role in the phototransduction cascade. Parallel studies on the *rd/rd cl* mouse employed action spectroscopy to characterise an opsin/vitamin A photopigment (OP) with a maximum sensitivity in the "blue" part of the spectrum (λ_{max} 479nm). Although the biochemistry of the photopigment had been deduced, the molecular identity of OP⁴⁷⁹ remained a mystery. Melanopsin is now known to form this photopigment. Melanopsin is expressed in the dpRGCs, and its genetic ablation in mice lacking all functional rods and cones abolishes circadian responses completely. The function of melanopsin has been assessed very recently by combining the expression of melanopsin protein with physiological assays of cellular photosensitivity. Remarkably, melanopsin can confer photosensitivity to a variety of non-photosensitive cell types. This presentation will review our recent and unpublished results relating to three areas: (1) Telemetry has been used to study both sleep and cardiac function in *rd/rd cl* mice. Our data show that non-rod, non-cone photoreceptors are capable of modulating both of these areas of physiology. (2) We have studied the development of the dpRGCs and provide direct evidence that they are light responsive from birth (P0) and this photosensitivity requires melanopsin. Interestingly, the number of dpRGCs at P0 is over 5 times that in the adult retina, reflecting an initial over-production of melanopsin-expressing cells during development. Even at P0, the dpRGCs form functional connections with the suprachiasmatic nucleus, as assessed by light-induced Fos expression. (3) Microarray experiments have been used to examine light-induced gene expression within the *rd/rd cl* eye. Our results show that approximately 20% of the transcriptome of the eye is regulated by non-rod/non-cone photoreceptors and we have identified a large number of candidate genes/proteins involved in the phototransduction cascade of the dpRGCs.

LIGHT, ENTRAINMENT AND THE ROLE OF CRYPTOCHROME IN SINGLE, LIGHT RESPONSIVE ZEBRAFISH CELLS

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The organs and tissues of adult zebrafish contain endogenous circadian clocks, which continue to run for many days *in vitro*. These tissue pacemakers can be reset directly by changing the lighting conditions within the incubator itself; an observation that has been extended to cell lines generated from zebrafish embryos. Using luminescent reporter gene technology, we are able to monitor circadian oscillations in a number of components of the central circadian clock. Single cell imaging approaches have allowed us to demonstrate that the clocks within these cells continue to run after many months in constant darkness. The phases of these cellular clock oscillations are widely distributed. However, a single 15-minute light pulse is sufficient to reset the clock to a common phase in the cycle. Recent work has strongly implicated the light induction of cryptochrome 1a as being essential in this strong resetting response. A variety of data supports this hypothesis, including manipulations of proteins that interact directly with CRY1a itself. Similar studies are being extended to look at clock function in the developing zebrafish embryo.

BLINDED BY THE LIGHT: EXORHODOPSIN IS REGULATED BY OTX5 AND PER3

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The pineal organ of zebrafish is an excellent model to study circadian rhythm because it encapsulates the entire circadian system. The pineal contains photoreceptors that entrain an endogenous circadian clock, and produces rhythmic outputs in melatonin and gene transcription. Our goal is to understand the regulation of rhythmic gene expression in the developing pineal. The transcription factor Orthodenticle homeobox-5 (OTX5) is required for expression of many rhythmic genes in the pineal. However, while OTX5 dependent genes are expressed in different phases, *Otx5* transcription is constitutive. Therefore, we hypothesize that OTX5 confers tissue specificity of gene expression, while rhythmic transcription factors regulate the phase. To test this, we have identified proteins that regulate expression of exorhodopsin (*Exorh*), a gene that encodes a pineal-specific photopigment. We find that *Exorh* is expressed in a strong rhythmic pattern when embryos are raised in a light/dark cycle, but expression becomes arrhythmic under constant conditions. Consistent with our model, we find that depletion of OTX5 causes loss of *Exorh* transcription in the pineal. In zebrafish, Period 3 (PER3) is a good candidate to be an OTX5 partner protein because it is expressed rhythmically throughout the central nervous system. Period proteins are components of the circadian clock that typically repress expression of circadian genes. In the absence of PER3, *Exorh* transcripts are no longer rhythmic, but constitutively high. Therefore, OTX5 activates pineal specific *Exorh* transcription, while PER3 exerts an oscillating, repressive effect. The murine system demonstrates that the circadian proteins PER 1 and 2 repress transcription by disrupting the positive transcription factors CLOCK and BMAL. While zebrafish *Per3* is circadian, we believe it does not function in the circadian clock. First, *Exorh* is not controlled by the circadian clock, because its rhythmic transcription is lost under constant conditions. Second, no binding sites for CLOCK/BMAL have been found in the *Exorh* promoter. Instead, PER3 could employ a unique method of transcriptional repression that is CLOCK/BMAL independent. To explore this idea, we examined the effects of *Per3* depletion on the circadian, CLOCK/BMAL regulated gene serotonin *N*-acetyl transferase 2. Loss of *Per3* had no effect. We speculate that PER3 operates in the phototransduction cascade to mediate the light-sensitive transcription of *Exorh*.

CIRCADIAN CLOCKWORK MACHINERY IN NEURAL RETINA: EVIDENCE FOR THE PRESENCE OF FUNCTIONAL CLOCK COMPONENTS IN CULTURED CHICK RETINAL CELLS

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Circadian clocks in chicken retina regulate a variety of biochemical and physiological processes. Retinal neurons, particularly photoreceptor cells, contain autonomous circadian clocks that control melatonin synthesis, iodopsin expression, *cFos* expression and cAMP synthesis. Photoreceptor enriched cell cultures, prepared from chick embryo retina and exposed to a daily light-dark (LD) cycle, exhibit a circadian rhythm of cAMP level. The present study was conducted to investigate melatonin synthesis and the expression of circadian clock genes in cultured retinal cells. In addition, the role of voltage-gated Ca^{2+} channels in the generation of the circadian rhythm of cAMP was investigated. Cultured cells exposed to LD showed rhythmic expression of clock genes. *Bmal1* and *mop4* peaked near the transition from light to dark. *Cry1* and *Per2* transcripts increased rapidly in the morning and were low at night. The rhythm of *Per2* was reduced in constant darkness (DD). *Clock* mRNA was high at night in LD, but arrhythmic in DD. Melatonin release and arylalkylamine *N*-acetyltransferase mRNA were low during day and high at night. The cells had a circadian rhythm of cAMP, with high levels at night. Nitrendipine, an L-type Ca^{2+} channel antagonist, abolished the circadian rhythm of cAMP. The Ca^{2+} channel agonist Bay K 8644 caused a significant increase in cAMP levels with continued circadian rhythmicity. The transcript encoding the Ca^{2+} -stimulated adenylyl cyclase AC1 was rhythmically expressed. These data suggest that a circadian clock controls the expression of *Ac1* and that calcium entry through voltage-sensitive calcium channels gates the circadian rhythm of cAMP, a key component in the regulation of retinal melatonin biosynthesis.

DO MW-CONES AND RODS CONTRIBUTE TO CIRCADIAN PHOTORECEPTION?

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In order to dissect out the role of different photoreceptors in clock mechanisms, we used a knockout (KO) mouse, which is characterized by an absence of MW cones coupled with an over-expression of SW opsin. Quantification of opsin content (MW, SW opsins, rhodopsin and melanopsin) was performed using real-time PCR in wild-type and KO mouse. Entrainment to light and light-induced phase shifts was assessed by monitoring locomotor activity in both groups of mice. Photoc entrainment was assayed using 3 levels of irradiances (1, 10 and 100 lux) combined with a 6 hour shift of the light-dark cycle. To measure light-induced phase shift, mice were exposed to two set of experiments: 1) 15 min of monochromatic light at three wavelengths (360, 480 and 530 nm) and 4 irradiance levels (10^{10} to 10^{14} photons/cm²/s) and 2) stimulation with 480 nm- light at one irradiance level and 3 durations (1, 5 and 15 min). Immunohistochemical labelling using specific antibodies against SW and MW opsins demonstrates the absence of MW cones and the over-expression of SW cones in KO mouse. Real time PCR confirms this result and also shows over-expression of melanopsin in the KO mouse. The rhodopsin content is similar in both strains. KO mice entrain to a light/dark cycle with reduced sensitivity compared to wild type mice. The KO mice show a robust phase-shift after a light pulse at 360 and 480nm; however, the magnitude of the response at 530 nm is significantly reduced compared to wild type mice. Light-stimulation (480 nm) at different durations shows that KO mice present a reduced sensitivity of the system compared to wild-type mice. These results demonstrate for the first time that MW cones and melanopsin contribute significantly whereas rods play a minor role in the photic signal that is transmitted to the circadian clock in the SCN.

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REGULATION OF MELANOPsin EXPRESSION BY LIGHT AND DARKNESS

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Circadian rhythms in mammals are daily adjusted to the environmental day/night cycle by photic input via the retinohypothalamic tract (RHT). Retinal ganglion cells (RGCs) of the RHT constitute a separate light detecting system in the mammalian retina which is used for irradiance detection to the circadian system and other non-imaging forming processes in the brain. The RGCs of the RHT are intrinsically photosensitive due to the expression of melanopsin, an opsin-like photopigment. This notion is based on anatomical and functional data and on observations in mice lacking melanopsin. Furthermore, heterologous expression of melanopsin in non-neuronal mammalian cell lines was found sufficient to render these cells photosensitive. Under such condition, melanopsin acts as a photopigment, coupled to a native ion channel via a G-protein dependent signalling cascade to drive physiological light detection. Even though solid evidence regarding the function of melanopsin exists, little is known about the regulation of melanopsin expression. We have examined whether the expression of melanopsin is regulated by an internal retinal clock and/or by the light/dark cycle. In the retina of albino wistar rats we observed diurnal changes of melanopsin mRNA and protein which seem to be driven by a biological clock. The diurnal changes in melanopsin expression seem, however, to be overridden by prolonged exposure to light or darkness. Significant increase in melanopsin expression was found already from the first day in constant darkness and the expression continued to increase during prolonged exposure in constant darkness. Prolonged exposure to constant light, on the other hand, decreased melanopsin expression to an almost undetectable level after five days of constant light. The induction of melanopsin by darkness was even more pronounced if darkness was preceded by light suppression for five days. Our observations show that dual mechanisms regulate melanopsin gene expression and that the intrinsic light responsive RGCs adapt their expression of the photopigment melanopsin to the environmental light and darkness.

THE NETWORK OF (AUTONOMIC) CLOCK OUTPUTS

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Despite the spectacular progress in elucidating the molecular mechanisms of circadian clocks, it is still not really understood how they lead to concerted output signals able to organize behavioral, physiological and hormonal rhythms. It is believed that circadian (locomotor) behavior is driven by humoral factors released from the SCN, but hormonal rhythms clearly depend on point-to-point axonal projections of the SCN neurons to specific target areas. So far, the most detailed information on the neural pathways employed by the SCN to transmit its rhythmic information is available for its circadian control of the hormones from the adrenal and pineal gland, i.e. corticosterone and melatonin, indicating an important role for the connections of the SCN with the autonomic nervous system. Both the adrenal and the pineal gland, however, mainly involve SCN control of the sympathetic part of the autonomic nervous system. In order to investigate if the circadian control of the SCN also comprises the parasympathetic part of the autonomic nervous system we extended our studies to the circadian control of glucose homeostasis. The daily rhythm in hepatic glucose production proved to be modulated by an SCN effect on sympathetic pre-autonomic neurons in the PVN as well. The activity of these pre-autonomic neurons is controlled by the combined effect of a continuous glutamatergic and a rhythmic GABAergic input from the SCN, i.e. very much similar to the control mechanism previously reported for the daily melatonin rhythm. In addition, however, our data revealed a further (organ specific) differentiation of SCN output, and a first glance at the possible involvement of peripheral oscillators. Moreover, when studying the effects of our hypothalamic manipulations on plasma insulin levels, i.e. a hormone from the endocrine pancreas, the first indications for a circadian control mechanism of the parasympathetic part of the autonomic nervous system were revealed.

INPUT AND OUTPUT PATHWAYS OF INSECT CIRCADIAN CLOCK NEURONS

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Neurons immunoreactive (-ir) to an antiserum against pigment-dispersing factor (PDF) are thought to be circadian pacemaker and output neurons in the cockroach *Leucophaea maderae* and other insects. In *L. maderae*, PDF-ir neurons densely arborise in the accessory medulla (AMe), a small neuropil of the optic lobe which is suggested to integrate timing information. The AMe is also conspicuously innervated by gamma-aminobutyric acid (GABA)-ir neurons, which are assumed to mediate light entrainment of the circadian clock. With double immunolabelling studies in *L. maderae* I could show that GABA is not a transmitter of PDF-ir neurons. On the ultrastructural level, GABA-ir processes were found synapsing on processes that morphologically resemble PDF-ir terminals. Currently it is investigated whether direct synaptic interactions between PDF-ir and GABA-ir neurons indeed occur in the AMe. In the central brain, PDF-ir neurons innervate large areas in which the target neurons for timing information are assumed to be located. To test whether these targets are descending neurons, tracer backfills of the cervical connectives of *L. maderae* were combined with anti-PDF immunolabelling. Only minor overlap of descending neurons with PDF-ir terminals was observed, thus suggesting that descending neurons are not the main effectors of the PDF-ir neurons in the cockroach. An alternative hypothesis proposes neurons of the pars intercerebralis (PI), which are integrated in neuroendocrine pathways, as effectors of the PDF-ir neurons. Since the myotropic neuropeptide leucokinin is present in the PI as well as in the AMe of *L. maderae*, double labellings with anti-leucokinin and anti-PDF were performed. Leucokinin-ir and PDF-ir processes overlap to some extent in the dorsal protocerebrum, thus suggesting that leucokinin-ir PI-neurons with dendrites in the dorsal brain could be part of the circadian output pathway in the cockroach. To reveal common features of insect circadian clocks, parts of this studies were extended to silverfishes (*Lepisma saccharina*) and *Drosophila*.

SUBSTANCE P IN THE HAMSTER RETINOHYPOTHALAMIC TRACT

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There has long been interest in the characteristics of the pathway through which photic information is transmitted from the retina to the circadian rhythm system. Elements of the pathway include melanopsin and non-melanopsin ganglion cells giving rise to the retinohypothalamic tract (RHT) and glutamate, thought to be the primary neurotransmitter of the RHT. There is also evidence of peptides in the RHT that function as neuromodulators of circadian rhythmicity. One of these is substance P (SP) which has been described as a constituent of the RHT in rat and hamster. However, there is substantial disagreement concerning its presence. We have examined the retina and brain of hamsters for the presence of SP cells and fibers. The data clearly show the presence of SP-IR cells in the whole mount retina. Moreover, there is a large degree of co-localization of SP-IR in ganglion cells also immunoreactive to the photopigment, melanopsin. Bilateral enucleation does not appear to change SP-IR fibers and terminals in the hamster SCN which has a known small population of intrinsic SP-IR neurons. Nor does enucleation eliminate the SP-IR terminal field in the hamster IGL. Despite the lack of apparent effect of bilateral enucleation on SP-IR retinal projections, retrograde tract tracer injected into the SCN demonstrates labeled retinal ganglion cells that are immunoreactive for SP and some of these also contain melanopsin-IR. Thus, immunohistochemical and retrograde tracing methods support the presence of SP in retinal ganglion cells contributing to the hamster RHT, but it is not clear whether the peptide is available for release from terminals of the RHT and post-synaptic effects on SCN function.

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CIRCADIAN ACTIVITY RHYTHMS AND PHASE-SHIFTING OF CULTURED NEURONS OF THE SUPRACHIASMATIC NUCLEUS OF THE RAT

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The mammalian suprachiasmatic nucleus (SCN) is the major endogenous pacemaker that coordinates various daily rhythms including locomotor activity, autonomous and endocrine responses through a neuronal and humoral influence. In the present study, we examined the behaviour of dispersed individual SCN neurons from 1- to 3-day-old rats cultured on multi-microelectrode arrays (MEAs) consisting of 60 microelectrodes of 30 μm in diameter. Extracellular spike activity of single SCN neurons cultured on MEAs at a density of ca. 3000 cells/ mm^2 was monitored over periods of up to 4 months. SCN neurons were identified by immunolabeling for the neuropeptides arginine-vasopressin (AVP) and vasoactive intestinal polypeptide (VIP). Single SCN neurons cultured at low density onto a MEA can express firing rate patterns with different circadian phases. In these cultures we observed only rarely synchronized firing patterns on adjacent electrodes. This suggests that in cultures of low cell densities SCN neurons function as independent pacemakers. In order to investigate the question whether individual pacemakers can be influenced independently by phase-shifting stimuli we applied for 30 minutes melatonin (100 nM), PACAP (100 nM) or glutamate (10 μM) at different circadian phases and continuously monitored the firing rate rhythms. All three substances elicited phase-shifting responses in individual clock cells which had no measurable input from other neurons. Phase-response curves (PRC) of single neurons differ somewhat from those previously described in brain slices. Phase-shifts of individual SCN neurons were also observed at times when the SCN showed no sensitivity to these phase shifting stimuli in recordings from brain slices. This suggests that the neuronal network plays an essential role in the control of phase-shifts.

POTENT CIRCADIAN EFFECTS OF DIM ILLUMINATION AT NIGHT IN HAMSTERS

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Conventional wisdom holds that the circadian pacemaker of rodents and humans is minimally responsive to light of the intensity provided by dim moonlight and starlight. Challenging this view, we have shown that provision of dim illumination (< 0.005 lux) during all dark periods markedly alters entrainment in hamsters: Under dimly lit scotophases, compared to completely dark ones, the upper range of entrainment is increased by ~4 h; re-entrainment is accelerated following transfer from long to short day lengths; and the incidence of bimodal entrainment to 24-h light:dark:light:dark cycles is increased fourfold. Notably, the nocturnal illumination inducing these pronounced effects is equivalent in photic energy to that of a 2 sec, 100 lux light pulse. In the present study, we demonstrate that the free-running pacemaker is also highly sensitive to this dim light. Syrian hamsters entrained to long or short photoperiods were released into constant darkness or constant dim light. Relative to complete darkness, dim illumination increased activity duration (alpha) by ~ 3 h and lengthened free-running period (tau) by ~0.3 h in each photoperiod group. Because amplitude of the bright light phase response curve is correlated with alpha, the increased range of entrainment under dim scotophases is likely a consequence of these changes in alpha. In concurrent experiments, discrete dim light pulses (≤ 2 h) produced statistically significant, but modest, phase shifts and melatonin suppression, indicating that these classic circadian responses to light are also more sensitive than previously appreciated. Together, the present results refute the view that scotopic illumination is environmental “noise.” To the contrary, clock function is markedly altered by nighttime illumination like that provided by dim moonlight and starlight. We interpret our results as evidence for a novel action of dim light on coupling of multiple circadian oscillators.

SUPRACHIASMATIC NUCLEI DIRECTLY REGULATE THE RHYTHM OF LEPTIN HORMONE RELEASE IN SYRIAN HAMSTERS (*MESOCRICETUS AURATUS*)

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The suprachiasmatic nuclei (SCN) generate the circadian rhythm of many hormones. Leptin hormone is a metabolic signal which informs the brain about fat and energy contents of the body. In the present study, we aimed to investigate whether the rhythm of leptin hormone release in Syrian hamsters is directly controlled by the SCN. Three experiments were performed; in the first, the hamsters were SCN-lesioned, in the second the hamsters were exposed to different feeding regimes (e.g., night-feeding animals were fed at day-time) and in the third, the hamsters were adrenalectomized and implanted with cortisol capsules to maintain a constant glucocorticoid release. Blood samples were collected before and after the experiments at different times of the day and examined for the leptin levels by ELISA. Different feeding regimes and constant glucocorticoid release did not change the rhythm of leptin release whereas SCN lesions resulted in an arrhythmic leptin release. The results of the present study showed that the rhythm of leptin hormone release is directly controlled by the SCN in the Syrian hamsters.

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CELL-AUTONOMOUS AND SYSTEMIC MECHANISMS IN CONTROLLING MAMMALIAN CIRCADIAN GENE EXPRESSION

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Circadian pacemakers were originally believed to exist only in a few specialized cell types, such as SCN neurons. However, in recent years, this view has been challenged by the discovery that self-sustained and cell-autonomous circadian clocks may exist in most peripheral organs. Peripheral oscillators are synchronized by humoral cues controlled by the SCN, such as glucocorticoids, or by physiological inputs such as food ingestion, food processing, or starvation. Indeed, the phase of peripheral clocks can be uncoupled from the SCN pacemaker by glucocorticoid hormones, feeding time, and temperature cycles. We are studying how peripheral oscillators can drive overt cycles in physiology. In principle, the positive and negative transcriptional regulators of the core clock circuitry can directly regulate the cyclic expression of effector genes, e.g. genes encoding enzymes.

Alternatively, circadian oscillators drive the rhythmic expression of positively and negatively acting transcription factors, which in turn regulate the cyclic expression of effector genes. We are studying two families of such output transcription factors, PAR bZip proteins and RORE-binding proteins. The PAR basic leucine zipper protein consists of the three members DBP, HLF, and TEF. Genetic and biochemical studies revealed that the expression of these transcriptional regulators is governed directly by core components of the molecular oscillator. In turn, the rhythmic accumulation of these transcription factors results in daily cycles of target gene expression. Transcriptome profiling experiments with knockout mice deficient for one, two, or all three PAR bZip proteins revealed that these transcription factors regulate mostly genes involved in hepatic and renal detoxification, and genes of an innate immune system. Indeed, PAR bZip deficient mice are exquisitely sensitive to many drugs, including medications used in anticancer therapy. The three PAR bZip proteins have partially redundant functions, as most target genes are downregulated much more dramatically in triple knockout mice than in single or double knockout mice. REV-ERB α , a nuclear orphan receptor acts as a strong transcriptional repressor when binding to RORE elements. It generates circadian transcription cycles of *Bmal1* and *Clock*, the two positive limb members of the molecular oscillators, by periodically repressing the transcription of these genes. In addition, it governs the circadian expression of several enzymes and regulators of lipid (triglycerides and cholesterol) metabolism. Our genetic Rev-Erb α loss-of-function and gain-of-function studies suggest that circadian transcription in the periphery can be governed by cell-autonomous and systemic cues.

A HIERARCHICAL NETWORK OF CLOCK REGULATES CIRCADIAN RHYTHMS IN THE MAMMALIAN RETINA

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Melatonin synthesis in mammalian retinal photoreceptors is under photic and circadian control and regulated by changes in the activity of arylalkylamine *N*-acetyltransferase (AA-NAT). Recent studies have suggested that retinal dopaminergic neurons contain a circadian pacemaker and dopamine is the neurotransmitter that is driving circadian rhythmicity in the mammalian retina. To investigate the role of inner retinal neurons, including dopamine neurons, in generating the rhythm of melatonin synthesis, rat retinas were lesioned with kainic acid (KA), which was shown previously to induce degeneration of neurons in the inner nuclear and ganglion cell layers, but does not kill photoreceptor cells. Our data show that intraocular injections of KA did not abolish the daily and circadian rhythms of *Aa-nat* mRNA in the photoreceptors, but it eliminated rhythmicity in the dopaminergic system. Surprisingly, we discover that KA injection eliminated the daily rhythm in *melanopsin* (*Opn4*) mRNA, thus indicating that dopamine is involved in the regulation of *melanopsin* mRNA. These findings demonstrate that the circadian clock generating melatonin rhythmicity is largely KA insensitive and likely to be located in the rod photoreceptors. More importantly our data demonstrate that dopamine rhythmicity is not necessary for generating the circadian rhythm of *Aa-nat* mRNA in the photoreceptors, but is necessary for the circadian rhythm in *melanopsin* mRNA.

CLOCKS HERE, CLOCKS THERE, CLOCKS EVERYWHERE? THE MOLECULAR CLOCKWORK SPARROW

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Birds are equipped with a complex circadian pacemaking system that regulates rhythmicity of physiology and behaviour. As with all organisms, transcriptional and translational feedback-loops of clock genes represent the basic molecular mechanism of rhythm generation in birds. To investigate whether clock gene expression is an exclusive property of well-characterised circadian oscillators, including the retina, the pineal gland, and the hypothalamus, we cloned partial cDNA sequences of six mammalian clock gene homologous (*pBmal1*, *pClock*, *pPer2*, *pPer3*, *pCry1* and *pCry2*) and a novel avian cryptochrome gene (*pCry4*). Expression patterns were analysed in various parts of the brain (telencephalon, diencephalon, cerebellum, tectum opticum) as compared to the retina and the pineal gland by semi-quantitative RT-PCR and RNase Protection Assay of total RNA extracted from adult male house sparrows. Levels of clock gene mRNA varied considerably between genes, tissues, and time points showing pronounced rhythmicity in all parts of the brain. In contrast to mammals where clock genes are oscillating out of phase with each other in different brain areas, most clock genes in the house sparrow brain oscillate in phase. Phasing of the various house sparrow clock genes when compared to each other as well as in relation to the light/dark cycle indicates that circadian control mechanisms in birds are highly distinct from mammalian model systems, both at the molecular as well as at the whole-organism level.

FINE TUNING OF PHOTOPERIODIC TIME INFORMATION IN AVIAN BRAIN

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The molecular mechanism underlying photoperiodic time measurement is not well understood in any organism. Japanese quail is an appropriate model system for the investigation of the photoperiodic time measurement because of their rapid and dramatic response of luteinizing hormone secretion to the photoperiod. Recently, we have found that the expression of type 2 deiodinase (*Dio2*), which catalyses the conversion of the prohormone thyroxine (T₄) to bioactive 3,5,3'-triiodothyronine (T₃), was up-regulated in the mediobasal hypothalamus (MBH) under long days. T₃ content in the MBH was about 10-fold higher under long days than short days and the intracerebroventricular infusion of T₃ under short days could mimic the photoperiodic gonadal response, suggesting the functional significance of *Dio2* expression in the MBH for the photoperiodic regulation of gonads. While DIO2 generates active T₃ from T₄ by outer ring deiodination, type 3 deiodinase (DIO3) catalyses the conversion of both T₃ and T₄ to inactive forms by inner ring deiodination. Therefore, DIO3 could play an important role in regulating appropriate active hormone concentrations locally. Expectedly, *Dio3* was densely expressed in the MBH under short days, whereas little expression was observed under long days. Moreover, after the transfer to a long day, *Dio2* was increased and *Dio3* was decreased from 16 hours after dawn. These gene changes precede the photoperiodic induction of luteinizing hormone secretion. These results suggest that the reciprocal gene switches regulate the functional thyroid hormone concentrations and trigger the photoperiodic response of gonads.

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INDUCTION OF *mPER1* mRNA EXPRESSION IN IMMORTALIZED GONADOTROPHS BY GONADOTROPIN-RELEASING HORMONE (GnRH): INVOLVEMENT OF PROTEIN KINASE C AND MAP KINASE SIGNALING

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The initiation and maintenance of reproductive function in mammals is critically dependent on the pulsatile secretion of GnRH. This peptide drives the pulsatile release of FSH and LH from the pituitary pars distalis via signaling pathways that are activated by the type I GnRH receptor (GnRH-R). Recently, a microarray analysis study reported that a number of genes, including *mPer1*, are induced by GnRH in immortalized gonadotrope cells. In view of these data, we have begun to analyze in detail the signaling pathways mediating the action of GnRH on *mPer1* expression in these cells. Using quantitative real-time PCR we could confirm that exposure of gonadotropes to the GnRH analogue, buserelin, markedly induces *mPer1* (but not *mPer2*) expression. Consistent with GnRH receptor signaling via the protein kinase (PK)-C pathway, exposure of the cells to phorbol 12,13-dibutyrate rapidly elevates both *mPer1* and LH β subunit mRNA levels, while pharmacological inhibition of PKC prevents the *mPer1* and LH β response to buserelin. As GnRH is known to regulate gonadotropin synthesis via activation of p42/44 mitogen-activated protein kinase (MAPK) signaling pathways, we then examined the involvement of this pathway in regulating *mPer1* expression in gonadotropes. Our data reveal that GnRH-induced *mPer1* expression was blocked following acute exposure to a MAPK kinase inhibitor. Although the involvement of this signaling mechanism in the regulation of *mPer1* is known in neurons, e.g. of the suprachiasmatic nuclei, the induction of *mPer1* in gonadotropes represents a novel mechanism of GnRH signaling, whose functional significance is still under investigation.

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REGULATION OF MELATONIN PRODUCTION IN FISH PHOTORECEPTOR CELLS

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The pineal organ of teleost fish contains cone-like photoreceptor cells connected to ganglion cells, interneurons, and supporting cells. The pineal photoreceptors share many structural and functional characteristics with the retinal photoreceptors, including the photoperiodic control of melatonin production. However, although ensuring similar functions, retinal and pineal photoreceptors each have their own sets of genes, perhaps as a result of a genome duplication that occurred at the base of the teleost fish lineage. Thus, teleost fish express two genes encoding each an arylalkylamine *N*-acetyltransferase (AANAT), the penultimate enzyme in the melatonin biosynthesis pathway. AANAT1 is retina-specific and AANAT2 is pineal-specific. Both enzymes are responsible for the rhythm in melatonin production despite the facts that (i) they display different kinetics and substrate specificity, and (ii) the strategies developed to regulate their rhythmic activity are different in each organ. The daily variations in AANAT activities are driven by day/night differences in ionic fluxes through the photoreceptor plasma membrane, which are triggered by the phototransduction process. In the dark, cyclic GMP (cGMP) accumulates and activates a type 3 cyclic nucleotide-gated channel (CNG3), allowing $\text{Na}^+/\text{Ca}^{2+}$ entry; the subsequent cell depolarization activates L-type voltage-gated Ca^{2+} channels, further increasing $[\text{Ca}^{2+}]_i$. Illumination induces, successively, cGMP degradation, closure of the CNG3 channels, cell hyperpolarization, closure of the L-type Ca^{2+} channels and a decrease in $[\text{Ca}^{2+}]_i$. In the pineal organ, the nocturnal accumulation of $[\text{Ca}^{2+}]_i$ favours an increase in cyclic AMP (cAMP) and both, Ca^{2+} and cAMP, contribute to increase AANAT2 protein abundance and activity. This probably involves phosphorylation of conserved residues in the AANAT2 protein. Both, protein amount and activity decrease upon illumination as a consequence of proteasomal proteolysis of the AANAT2 protein. The effects depend on the wavelength and light intensity, as is the case for the photoreceptor membrane potential. This translational/post-translational regulation of AANAT2 exists in all teleost fish investigated. The photoperiodic control of AANAT2 may also involve transcriptional regulation of the *Aanat2* gene (except in trout where *Aanat2* is constitutively expressed). This involves a circadian clock located within the photoreceptor cells, and the *Aanat2* gene is a direct output of this clock. Light input to the clock is mediated by the induction of the transcription factor PER2, through a yet unknown mechanism. In brief, morning light induces an immediate reduction in AANAT2 protein amount and activity, and an increase in *Per2* transcription. PER2 is necessary for the following increase in *Aanat2* expression and subsequent nocturnal increase in AANAT2 protein amount and activity. The amplitude of this nocturnal rise is modulated by temperature and internal factors (hormones, neurotransmitters, neuromodulators). Thus, it is the combined action of several factors which determines the shape of the melatonin oscillations, and these oscillations vary along the annual cycle. The retina shows striking differences with the pineal organ. First, not only photoreceptor cells, but also cells in the inner nuclear and ganglion cell layers synthesize melatonin. Second, AANAT1 protein amount, AANAT1 activity and melatonin concentration increase during day not at night. Because retinal melatonin acts and is metabolized in situ, this reversed pattern may reflect specific adaptations to retinal fish physiology.

ZEBRAFISH *Period2* EXPRESSION PATTERN AND ITS ROLE IN THE DEVELOPMENT OF THE CIRCADIAN CLOCK

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In zebrafish, pineal arylalkylamine-N-acetyltransferase (*zfaanat2*) mRNA expression begins at 22 hour post-fertilization (hpf) and a clock-controlled rhythm of its transcript begins on the third day of development. Here we describe the role of light and of the clock gene, *period2* (*zper2*), in the development of this rhythm. In one-day-old zebrafish embryos, *zper2* expression is transiently up-regulated by light in the pineal gland, and, to lesser extent, in other areas of the brain. Expression of *zper2* that was less affected by light occurred in the olfactory placode and lactotroph cells of the pituitary primordium. Circadian analysis of pineal *zfaanat2* mRNA expression indicated that light exposure is required for proper development of the circadian, clock-controlled, rhythmic expression of *zfaanat2*. Knock-down of *zper2*, using antisense technology, abolished the *zfaanat2* mRNA rhythm, corroborating the link between the light-induced *zper2* expression and the light-induced *zfaanat2* rhythm. Further analysis of *zper2* expression at earlier stages of development revealed that light treatments at the blastula stage to mid-somatogenesis also causes a transient increase in *zper2* expression. This expression was ubiquitous at first, but as development proceeded, it was enhanced in the midline and lateral edge of the neural plate, regions which give rise to the diencephalon, olfactory bulb and pituitary. At the stage of neural tube formation, the bilateral *zper2* signals at the posterior edge of the anterior neural plate converged dorsally and fused at the midline where the pineal gland develops. Thus, light induces significant *zper2* expression in embryonic cells that later form the pineal gland, olfactory bulb and pituitary. The expression of *zper2* in the olfactory and pituitary primordium reflects its possible involvement in life-long time-keeping mechanisms. The early photoreception and light-induced *zper2* expression may be important for the establishment, or onset, of the circadian clock.

THE AVIAN PINEAL GLAND

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The pineal gland plays a key role in control of the circadian and seasonal rhythms in most vertebrate species. In mammals, the rhythmic melatonin release from the pineal gland is controlled by the CNS, primarily by the suprachiasmatic nucleus, via the sympathetic system. In most non-mammalian species, including birds, the pineal gland possesses high degree of autonomy containing a fully functional circadian biological clock and several input channels, including direct light sensitivity, which synchronize the clock. The avian pineal gland survives well under in vitro conditions and maintains its rhythmic activity for days. Several physical (light, temperature, magnetic field) and biochemical (VIP, norepinephrine, PACAP, etc.) input channels, entraining circadian rhythmic release of melatonin, are also functional in vitro rendering the explanted avian pineal an excellent model of the circadian biological clock. Mostly based of our experiment on explanted chicken pineal glands, a functional dissection of the control and mechanism of the rhythmic events in the avian pineal gland will be presented.

A NEW WAY OF REGULATING RODENT *Aanat* GENE EXPRESSION, EXAMPLE OF THE SYRIAN HAMSTER

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The Syrian hamster is a rodent in which photoperiodic changes in the duration of the melatonin peak are pivotal for the synchronisation of seasonal functions like reproduction. Interestingly, we recently found that the activity of *N*-acetyltransferase (AANAT), the key enzyme for rhythmic synthesis of melatonin, is precisely controlled and gated in the Syrian hamster, suggesting a different regulation than that of the rat or mouse. During the night, the late *Aanat* gene expression is preceded by a rapid and transient norepinephrine-dependent phosphorylation of CREB and synthesis of the immediate early gene products c-FOS and c-JUN that are peaking 3 hours after dark onset. c-FOS synthesis requires both the beta/alpha-adrenergic receptor pathway and the p-ERK 1/2 pathway. Interestingly, injection of the protein synthesis inhibitor cycloheximide inhibited *Aanat* mRNA expression only if injected before the c-FOS/c-JUN peak. This suggests that the c-FOS/c-JUN dimer is required for transcriptional activation of *Aanat* gene. During the day, *Aanat* gene expression and therefore melatonin synthesis are blocked. The inhibitory transcription factor ICER is present in the pineal gland but is peaking when AANAT may be activated, suggesting that the blockade takes place upstream of *Aanat* gene expression. Preliminary experiments indicate that the diurnal inhibition of AANAT occurs at the level of the adrenergic receptor signalling pathway. These findings demonstrate that AANAT regulation in the Syrian hamster requires a complex intracellular signalling different from that described in the rodents like mice and rats.

OPPOSITE EFFECTS OF PROTEASOME INHIBITORS ON THE ADRENERGIC INDUCTION OF ARYLALKYLAMINE *N*-ACETYLTRANSFERASE IN RAT PINEALOCYTES

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We have investigated the effect of proteasomal inhibition on the induction of arylalkylamine *N*-acetyltransferase (AANAT) enzyme in cultured rat pinealocytes, using two proteasome inhibitors, MG132 and clasto-lactacystin beta-lactone (c-lact). Addition of c-lact or MG132 3 hours after norepinephrine (NE) stimulation produced a significant increase in AANAT protein level and enzyme activity as previously reported. However, when the proteasome inhibitors were added prior to or together with NE, significant reductions of the NE-induced *Aanat* mRNA, protein and enzyme activity were observed. A similar inhibitory effect of MG132 on *Aanat* transcription was observed when cells were stimulated by dibutyryl cAMP, indicating a post-cAMP site of action. The inhibitory effect of MG132 on adrenergic-induced *Aanat* transcription was long lasting because it remained effective 14 hours after washout, and selective because the induction of mitogen-activated protein kinase phosphatase-1 (mkp-1) by NE was not affected. Time profile studies revealed that the inhibitory effect of MG132 on NE-stimulated *Aanat* induction was detected after 1 hour suggesting accumulation of a protein repressor as a possible mechanism of action. This possibility was also supported by the finding that the inhibitory effect of c-lact on NE-induced *Aanat* induction was abolished by cycloheximide, a protein synthesis inhibitor. Together, these results suggest that proteasome activities are required to prevent the accumulation of a protein repressor that in turn may suppress the adrenergic-mediated induction of *Aanat* transcription.

THE PERIVASCULAR PHAGOCYTES OF THE PINEAL GLAND: AN ANTIGEN PRESENTING CELL

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The mammalian pineal gland consists of a parenchyma separated by connective tissue septae containing blood vessels. The cellular content of the parenchyma comprises: a) melatonin secreting pinealocytes, and b) the darker stained interstitial cells. In addition, a third cell type, the perivascular phagocyte, has been described during the last decade. The perivascular phagocyte is located in the perivascular spaces close to pineal capillaries, which are without a blood-brain barrier. These cells are endowed with phagocytic properties, as indicated by a prominent uptake of tracers into intracellular vesicles and lysosomes. The perivascular phagocyte expresses marker proteins for the macrophage/monocyte cell line (Mukda et al., *Eur. J. Neuroscience*, 21: 2743-2751, 2005) and MHC class II proteins (Pedersen et al., *Cell Tissue Res.*, 272:257-65, 1993), thereby defining the phagocytes as antigen presenting cells. These antigen-presenting cells might be involved in autoimmune responses involving proteins expressed at high levels in the pineal gland and retina. For example, the S-antigen (retinal arrestin), is a highly pathogenic autoantigen that can induce experimental autoimmune uveitis and pinealitis (Pineal and Retinal Relationships. O'Brien, P.J. and Klein, D.C. (eds) Academic Press, Orlando, pp. 343-355, 1986; Donoso et al., *Curr Eye Res.* 9 Suppl:155-61, 1990). Perivascular phagocytes could participate in this through uptake of pinealocyte-derived S-antigen followed by immediate presentation to CD8+ T cells in the gland. However, if the S-antigen is phagocytosed by the perivascular cell without being presented by the MHC II system on the surface, these cells might function as a sentinel system reducing the risk of autoimmune responses.

DAILY OSCILLATION IN MELATONIN SYNTHESIS IN THE TURKEY PINEAL GLAND AND RETINA: DIURNAL AND CIRCADIAN RHYTHMS

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Although rhythmic melatonin (MEL) production has been studied in the pineal glands of several birds, the MEL-generating system in the avian retina has to date only been investigated in a few species. In the present study MEL synthesis was examined in the pineal gland and retina of the turkey, an avian species whose physiological processes are controlled by the daylength. The turkey pineal gland and retina rhythmically produced MEL. In animals kept under a daily light: dark (LD) illumination cycle MEL concentrations in the pineal gland and retina were low during the light phase and high during the dark phase. A rhythmic oscillation in MEL, with high night-time values, was also observed in the plasma. The pineal and retinal MEL rhythms reflected oscillations in the activity of serotonin *N*-acetyltransferase (AANAT; the penultimate and key regulatory enzyme in MEL biosynthetic pathway). In contrast, in both tissues the activity of hydroxyindole-*O*-methyltransferase (HIOMT) did not exhibit significant changes throughout the 24-h period. Acute exposure of turkeys to light at night (150 lux for 30 min at ZT 18) dramatically decreased MEL levels in the pineal, retina, and plasma. Rhythmic oscillations in AANAT activity and MEL concentrations in the turkey pineal gland and retina were circadian in nature as they persisted under conditions of constant darkness (DD). Under DD, however, the amplitudes of AANAT and melatonin rhythms were significantly lower (by 50-80%) than those found under the LD cycle. These findings indicate that MEL rhythmicity in the turkey pineal gland and retina is regulated by light and the endogenous circadian clock. The rapid dampening of the rhythms under DD suggests that of these two regulatory factors, environmental light may be the primary stimulus in the maintenance of the high amplitude MEL rhythms in the turkey.

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PHOTIC REGULATION OF AN ARYLALKYLAMINE *N*-ACETYLTRANSFERASE / 14-3-3 COMPLEX IN THE CHICKEN RETINA

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Light exposure rapidly inhibits arylalkylamine *N*-acetyltransferase activity and melatonin synthesis in retina and pineal of most vertebrate species. The mechanism of AANAT regulation by light was examined in the chicken retina. Light exposure significantly increased the apparent K_m of AANAT for its substrate, tryptamine. During gel filtration chromatography, AANAT activity eluted as three peaks with different apparent molecular weights (MW). A low MW fraction corresponded to the free, monomeric enzyme. A second peak of activity was identified as an AANAT/14-3-3 complex. The K_m of the AANAT/14-3-3 complex for tryptamine was 0.02 mM, which is approximately 15 times lower than the K_m of the free enzyme. The physical nature of high MW AANAT is unknown, but it may represent enzyme that is docked to the proteasome. In dark adapted retinas, most of AANAT exists as a complex with 14-3-3 proteins. Unexpected light at night caused dissociation of AANAT/14-3-3 complex, and an increase in high MW form of enzyme when proteasomal proteolysis was inhibited by lactacystin. An incubation of protein extract with catalytic subunit of protein kinase A favored the formation of AANAT/14-3-3 complex. Using AANAT specific phospho- and non-phosphopeptides, we were able to show that the phosphorylation of Thr-29 and Ser-203 is important for the binding of enzyme to 14-3-3 proteins. Our results are consistent with the hypothesis that AANAT is phosphorylated in the dark, favoring the binding to 14-3-3 proteins. This interaction protects the enzyme from degradation and increases its affinity for substrate. Upon light exposure, the enzyme dissociates from 14-3-3, is inactivated and dephosphorylated, and is targeted for degradation by the proteasome.

CHARACTERIZATION OF DIURNAL PATTERNS IN *Aanat* mRNA, AANAT ACTIVITY AND MELATONIN CONTENT IN AUTOPTIC HUMAN PINEAL TISSUE

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The mammalian pineal organ is a peripheral oscillator, in which observed rhythms depend on afferent information that is derived from the endogenous clock residing in the hypothalamic suprachiasmatic nucleus. The best characterized function of the pineal gland is the rhythmic synthesis of the hormone melatonin, which provides the body with the signal of the duration of the night period. The rate-limiting enzyme for melatonin synthesis is the arylalkylamine *N*-acetyltransferase (AANAT). In contrast to the transcriptional regulation of the *Aanat* gene in rodents, a post-translational shaping of the melatonin pattern is indicated in the human pineal gland. To gain further insight into the rhythmic processes involved in melatonin synthesis, we collected human pineal tissue from regular autopsies. Out of all samples only those were processed further, in which time of death could be established within an error range of four hours (n=58). Expression of *Aanat* in pineal tissue was examined with PCR, using a Gene-Bank-derived primer pair. AANAT-activity was measured using radioactively labelled acetyl-coenzyme A, and pineal melatonin concentrations were analyzed with an ELISA. Despite large postmortem intervals of up to 144 hours, it was possible to gain high quality RNA and protein out of most samples. It could be shown that the RNA degradation rates of genes of interest run in parallel and, therefore, data normalization could be established, irrespective of postmortem delay in tissue sampling. The semi-quantitative analysis of *Aanat* mRNA showed no diurnal rhythm. Measured AANAT-activity and melatonin concentrations were highest between 10 p.m. and 5 a.m. Data support for the first time experimentally that post-translational mechanisms are responsible for the generation of rhythmic melatonin synthesis in the human pineal gland.

AGE-RELATED CHANGES IN THE CIRCADIAN AND HOMEOSTATIC REGULATION OF HUMAN SLEEP

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In healthy aging, the reduction of both hallmarks of human non-REM sleep - EEG slow-wave activity and sleep spindles - leads to less consolidated sleep. Whether this reduction is due to circadian or sleep homeostatic mechanisms is not yet known. We have assessed circadian and homeostatic parameters in 16 healthy young (20-31 y) and 16 older volunteers (57-74 y) under high - and low sleep pressure conditions in a 40-h constant posture protocol. We observed an age-related reduction in the amplitude of the endogenous circadian component of the melatonin rhythm of about 40%, a reduced circadian modulation of REM sleep together with a less pronounced day–night difference in the spindle range of sleep EEG activity. This could be interpreted as lowered output of a weaker circadian arousal signal opposing the build-up of homeostatic sleep pressure during the wake episode in the older volunteers. More sleep occurred during the wake maintenance zone, and older subjects experienced higher subjective sleepiness in the late afternoon and evening. There was no age difference in the relative increase in EEG power density in the delta range after 40 h of sleep deprivation, indicating a sustained capacity of the sleep homeostat to respond to sleep loss in aging. The increase in relative EEG delta activity following sleep deprivation showed a frontal predominance (compared with parietal brain regions) in the young, which was diminished in the older volunteers. These findings provide quantitative evidence for the hypothesis that frontal brain regions are particularly vulnerable to the effects of both elevated sleep pressure and aging. In conclusion, our data suggest that potential manipulations of the circadian timing system rather than the sleep homeostat may offer a potential strategy to alleviate age-related changes in sleep and daytime alertness levels.

AGE-RELATED EFFECTS ON THE BIOLOGICAL CLOCK AND ITS BEHAVIOURAL OUTPUTS IN A PRIMATE

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In humans, activity rhythms become fragmented and attenuated in old people. This suggests an alteration of the circadian system per se, that could in return affect the expression of biological rhythms. In primates, very few studies have looked at the effect of aging on the circadian system. The mouse lemur provides a unique model of aging in non-human primates. To assess the effect of aging on the circadian system of this primate, we recorded the circadian and daily rhythms of locomotor activity of mouse lemurs of various ages. We also examined age-related changes in the daily rhythm of vasoactive intestinal polypeptide (VIP) and arginine-vasopressin (AVP) expression in suprachiasmatic nucleus (SCN) neurons, two major peptides of the biological clock. Compared to adult animals, aged mouse lemurs showed a significant increase in fragmentation and an advanced activity onset. Aging was characterized by a decreased amplitude, with both a decrease in nocturnal activity and an increase in daytime activity. These changes are similar to the age-related changes in rest-activity rhythm observed in humans. Moreover, when maintained in constant dim red light, aged animals exhibited a shortening of the free-running period compared to adult animals. Phase of entrainment to light was dependent of individual period values. In adult animals, AVP immunoreactivity peaked during the second part of the day, and VIP peaked during the night. In aged mouse lemurs, the peaks of AVP and VIP were significantly shifted. AVP was most intense at the beginning of the night, whereas VIP peaked at the beginning of daytime. A weakened oscillator could account for the rhythmic disorders often observed in old subjects. Changes in the daily rhythms of these two main peptidergic cell populations may affect the SCN ability to transmit rhythmic information to other neural target sites, and thereby modify the expression of some biological rhythms.

THE AGING SUPRACHIASMATIC NUCLEUS AND CYTOKINES: MOLECULAR, CELLULAR AND FUNCTIONAL CHANGES IN RODENTS

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Senescence leads to a switch to a chronic low-grade proinflammatory status at the cellular and molecular levels, with increased production of cytokines in the organism, including the brain. It is also well known that the regulation of endogenous biological rhythms, controlled by the hypothalamic suprachiasmatic nuclei (SCN), is altered during aging. Such changes, and especially alterations of the sleep/wake cycle, notably affect the health and quality of life in the elderly. To unravel neurobiological correlates of such changes, we investigated the SCN in old mice compared to young ones. Electrophysiological properties of the SCN in slices showed marked reduction in the frequency of spontaneous GABAergic transmission in the SCN core (the retino-recipient region) in old mice. Furthermore, day/night rhythm was seen in the proportion of silent neurons in the SCN core of young mice but not in old ones, in which such proportion was high during the day. The study of the circadian variation of transcripts encoding cytokine receptors and suppressors of cytokine signaling (SOCS) was combined with analysis of Fos induction elicited in SCN neurons by intracerebroventricular administration of the cytokines interferon-gamma and tumor necrosis factor α . This part of the investigation pointed out marked Fos induction in SCN neurons at early night, coinciding with low *Socs1* and *Socs3* expression. In the aged SCN, such Fos induction was reduced, with differences in the sub-regional prevalence of Fos-labeled neurons, and was paralleled by decreased expression of interferon-gamma receptor. The study of SCN glia in response to the same inflammatory challenge showed stronger activation of astrocytes and microglia in the aged than in the young SCN, with variation of sub-regional prevalence of activated astrocytes within the nucleus. Altogether the data point out marked changes in the aged SCN, including disturbances in neuroimmune signalling that could also affect the intra-SCN signalling during aging.

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CHANGES OF THE GABAERGIC NETWORK IN THE SUPRACHIASMATIC NUCLEUS OF AGED MICE

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The inhibitory neurotransmitter GABA is present in the majority of neurons in the suprachiasmatic nucleus (SCN), the main circadian pacemaker in the mammalian brain. GABA plays an important role in synchronizing the neuronal network but little is known about the function of the GABAergic network in individuals during aging, when, however, biological rhythm disturbances are common. We here investigated GABAergic synapses in the SCN, comparing young (2-3 months) and aged (18-21 months) C57B/6J male mice, maintained under a light/dark cycle. Double immunofluorescence and confocal microscopy were used to investigate presynaptic GABAergic elements in the SCN. Glutamic acid decarboxylase (GAD; isoform 67), the rate-limiting enzyme for GABA synthesis, was used as marker for GABAergic terminals; synaptophysin, a synaptic vesicle protein, was used as general marker for presynaptic terminals. Quantitative image analysis of labelled areas was performed in the core part of the SCN. Old mice displayed a significant reduction compared to young mice in the total synaptophysin-labelled area (all terminals), as well as in the total area of colocalized GAD and synaptophysin labelling (GABAergic terminals) in the SCN core. Analysis of these parameters in the SCN shell is at present in progress. In addition, we examined, using real-time PCR, the daily expression of the alpha3 subunit of the GABA_A receptor, which is located both pre- and postsynaptically in the SCN. The expression of this receptor exhibited significant day/night variation in young mice, with a peak in the early night, whereas no significant difference was found between different time points in old mice. The present findings of a decrease of GABAergic terminals and lack of daily oscillation of the alpha3 subunit of the GABA_A receptor in the aged SCN suggest a dysregulation of inhibitory neurotransmission, which could result in changes of circadian function.

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ALTERATION OF DAILY URINE 6-SULFATOXYMELATONIN, PLASMA CORTICOSTERONE AND HEPATIC CLOCK GENE EXPRESSION IN THE FOLATE DEFICIENT CBA MOUSE

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Folate is a cofactor of CRY protein and is involved in methylation of many biological compounds including *N*-acetyl-tryptamine, the precursor of melatonin. These data prompted us to study in the mouse the influence of folate deficiency on daily melatonin production, plasma corticosterone (a marker of the circadian clock) and expression of clock genes in a peripheral tissue, the liver. Eight-week-old CBA mouse submitted to a 12/12 light/dark cycle were fed an identical diet with or without folate (25 animals in each group). At the end of a 4 week treatment, 12h urine samples (8h-20h, 20h-8h) were collected. After housing for 24h in complete darkness to eliminate the possible masking effect of light on clock gene expression, the mice were hourly anaesthetised and killed under red light. Blood and organs were collected. Plasma folate and corticosterone and urine 6-sulfatoxymelatonin (aMT6s) were determined by radioimmunoassay. Hepatic RNAs were extracted with RNABle (Eurobio^R) and quantified using real time PCR (Light Cycler Roche^R). Compared with controls, all animals fed the folate deficient diet showed a decreased erythrocyte folate concentration ($p < 0.0001$), a decrease in day and night urine aMT6s elimination ($p < 0.05$ for both), and an advanced phase for the 24h plasma corticosterone profile. A 2 way-ANOVA followed by multiple comparisons performed on the daily clock gene expression showed a phase-advance for *Cry1*, *Per1* and *Per3* expressions, a major increase of *Per2* expression during the overall subjective night, and an increase of *Cry1*, *Clock*, and *Bmal* expressions at the end of the subjective night. The results obtained for aMT6s excretion in the CBA mice confirm those obtained in folate deficient rats. Taking into account the alterations of both folate metabolism and rhythms (decreased amplitude and advanced phase) observed in old people, we suggest that the folate deficient CBA mice could be a model of aging.

PHOTIC SENSITIZATION IN AGED HUMANS

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TRANSCRIPTIONAL CONTROL OF THE MAMMALIAN CIRCADIAN CLOCKWORK

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Genetic, molecular and biochemical approaches have revealed that the mouse circadian clockwork is composed of interacting positive and negative transcriptional feedback loops, which drive recurrent rhythms in the RNA and protein levels of key clock components. Recent studies have revealed additional transcriptional control mechanisms.

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THERAPEUTIC POTENTIAL OF MELATONIN LIGANDS

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Melatonin is a neurohormone which has been claimed to be involved in a wide range of physiological functions. In humans, appropriate clinical trials have only confirmed the efficacy of melatonin or melatonergic agonists for the MT1 and MT2 receptor subtypes in circadian rhythm sleep disorders. Nevertheless, preclinical studies in animal models, relevant to human pathologies and validated by reference compounds, opened other therapeutic perspectives. Among those, a new concept in the treatment of depression involving MT1 and MT2 agonist and a 5-HT_{2C} antagonist activities has been recently proposed and clinically validated by agomelatine. A third melatonin binding site has been purified and characterized as the enzyme quinone reductase 2. The physiological function of this enzyme is not known, but recent results obtained by different groups suggest that the inhibition of this enzyme may lead to a protective effect and its overexpression to a deleterious effect. The inhibitory effect of melatonin on quinone reductase 2 observed in vitro may explain the protective effects reported for melatonin in different animal models, such as cardiac or renal ischemia and attributed to the controversial anti-oxidant properties of melatonin. Lastly, the orphan receptor GPR50, also named Melatonin Related Receptor, must not be forgotten. This receptor shares the unique features of the melatonin receptor group, but surprisingly, does not bind melatonin. A link between a polymorphism in GPR50 and bipolar affective disorder in women has been recently reported. The development of specific ligands for each of these melatonin binding sites is necessary to link a physiological function and/or a therapeutic effect to a specific binding site.

ARE BOTH MT1 AND MT2 RECEPTORS INVOLVED IN MELATONIN-MEDIATED PHASE SHIFTS OF CIRCADIAN RHYTHMS?

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In mammals, melatonin phase shifts circadian rhythms through receptor-mediated mechanisms. This presentation will describe potential mechanisms by which activation of MT1 and/or MT2 receptors phase shifts circadian rhythms of neuronal firing in the mouse suprachiasmatic nucleus brain slice and of overt circadian rhythms in vivo. Melatonin phase advances (CT10) circadian rhythms of neuronal firing in the suprachiasmatic nucleus brain slice from wild type and MT1 knockout but not MT2 knockout mice. Furthermore, the phase shifts of neuronal firing mediated by melatonin were blocked by MT2 selective concentrations of the competitive melatonin receptor antagonist 4P-PDOT. Paradoxically, administration of melatonin in vivo phase advances the onset of circadian rhythms of activity in the wild type but not in the MT1 knockout mouse, requiring MT1 receptor activation for in vivo phase shifts. These results will be discussed in relation to molecular events regulated through activation of MT1 and MT2 melatonin receptors within and outside the suprachiasmatic nucleus that may be necessary for expression of phase shift of overt circadian rhythms.

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MODULATION OF MELATONIN RECEPTOR FUNCTION BY RECEPTOR DIMERIZATION AND SCAFFOLDING PROTEINS

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In mammals, the circadian hormone melatonin targets two seven trans-membrane spanning receptors, MT1 and MT2 that activate G_i and G_q associated signaling pathways. We show that these receptors exist as homodimers and may form heterodimers when co-expressed in the same cell. Whereas MT1 is equally distributed into homo- and heterodimeric complexes, MT2 is preferentially engaged in MT1/MT2 heterodimers with distinct ligand binding properties that are different from MT2 homodimer. Intracellular signaling events are often organized around PDZ domains containing scaffolding proteins in neurons. MT1 contains a putative PDZ domain binding motif in its carboxy-terminal tail (DSV) that is absent in MT2. We show here that this motif is functional and that binding of PDZ domains to this motif can participate in the fine-tuning of receptor function. These results show that melatonin receptor function is modified depending on the co-expression of molecular interaction partners.

THE SUPRACHIASMATIC NUCLEUS: MULTIOSCILLATOR STRUCTURE WITH DIFFERENT PHASE RESETTING UNITS

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Neurons of the suprachiasmatic nucleus (SCN) of mammals have a genetic basis for rhythm generation, resulting in rhythmic discharge patterns in individual SCN neurons. We performed recordings of electrical impulse frequency in SCN slices with stationary electrodes. Recordings of large populations, consisting of over 100 neurons, revealed consistent peaks in electrical discharge patterns during the mid subjective day (CT6). Recordings of small subpopulations revealed that subpopulations peak at various phases of the subjective day. Single unit activity recordings revealed an even larger variation in peak time, and showed that individual neurons show surprisingly short periods of elevated electrical activity. The sum of the single or subpopulation activity patterns accounts for the multiunit discharge pattern with a consistent peak at CT 6 (Schaap et al, 2003). To understand phase resetting of the circadian system we performed recordings in SCN slices in response to a 6h shift of the light cycle. Following a delay of the cycle, we observed bimodal patterns in electrical activity in the SCN. The bimodal patterns were observed both in the ventral as well as in the dorsal SCN. One of the peaks corresponded with the unshifted electrical activity rhythm while the secondary peak occurred at the new CT6. When we bisected the SCN, we found a unimodal shifted rhythm in the ventral SCN and an unshifted rhythm in the dorsal SCN. We conclude that in intact slices, the ventral and dorsal SCN communicate, and impose their electrical activity on the other half of the SCN (Albus et al, 2005). In vivo electrophysiological recordings revealed slow phase resetting responses to advances but not to delays of the light cycle. These differences between advancing and delaying responses were not observed in the isolated SCN, in slices. We obtained evidence that extra-SCN areas retard phase advancing responses of the SCN (Vansteensel et al, 2003).

CIRCADIAN CLOCKWORK AND ENTRAINMENT DURING DEVELOPMENT

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The rhythmicity of the central circadian clock within the suprachiasmatic nuclei (SCN) is driven by a molecular clockwork. Morphologically, the SCN develops already during embryonic stage, however, synaptogenesis is not completed until early postnatal ontogenesis. The connection between the SCN and retina develops from postnatal day 1 (P1) till P10. The SCN clock output rhythmicity is already present in the late embryonic stage. The aim of the present study was to ascertain when and how the rat SCN molecular clockwork develops functionally during pre- and postnatal ontogenesis. We studied (i) the development of the circadian profiles of clock gene mRNAs in the rat SCN at embryonic days (E)19 and E20, and at P1, P2, P3 and P10, (ii) the effect of absence of a light-dark cycle on the pre- and postnatal development of the circadian profiles of clock gene mRNAs, (iii) development of circadian control of the clock gene photosensitivity, and (iv) development of photoperiodic entrainment of the circadian profiles of clock gene mRNAs during early postnatal ontogenesis. The circadian rhythmicity of clock gene mRNA levels was not yet present at E19 and E20 but occurred at P1 and then gradually developed until P10. The dynamic of the development was not affected by absence of a light-dark cycle. The photosensitivity and photoperiodic entrainment developed gradually until P20. In summary, the data indicate an endogenous gradual development of functional properties of the circadian clock during postnatal ontogenesis.

HAMSTERS RUNNING ON TIME: IS THE HABENULA A PART OF THE CLOCK?

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While the lateral habenula is intimately associated with the pineal gland in lower vertebrates, it generally has not been considered to be part of the circadian system in mammals. Nevertheless, it is already known to be innervated by the suprachiasmatic nucleus (SCN) in rodents, to exhibit a circadian rhythm of firing rate *in vivo* and *in vitro* in rats, and to express high-affinity melatonin receptors in golden hamsters. Here we report a remarkable and unexpected asymmetry of c-Fos expression in the medial division of the lateral habenula of ‘split’ hamsters in constant light (in which the right and left sides of the SCN oscillate in antiphase), but only during the active phase (when the animals are running in their wheels). High c-Fos levels are ipsilateral to the split SCN’s subjective-night side, just as high levels are present in normal, unsplit hamsters during subjective night in constant darkness. The function of the lateral habenula is not certain, although it has been hypothesized to be involved in the control of movement by motivational stimuli. Importantly, locomotor activity rhythms do not appear to be driven by a unitary circadian output mechanism. In a model proposed by Davis and Menaker (1980) for hamsters, there is a ‘window’ oscillator output that determines a time for activity (subjective night) and a time for rest (subjective day), interacting with a ‘bout’ oscillator output that controls the expression of locomotion *per se*. We suppose that one of these outputs might be diffusible, and we speculate that the other might be neural, with lateralized axonal projections involving the lateral habenula.

CYTOKINES ALTER GABA-ERGIC POSTSYNAPTIC ACTIVITY IN SUPRACHIASMATIC NUCLEUS NEURONS

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Cytokines have been ascribed somnogenic properties. Little is known about the effects of cytokines on circadian rhythms. The pro-inflammatory cytokine interferon-gamma (IFN- γ) is an immunomodulatory molecule released by immune cells and was originally described as an anti-viral agent. Previous studies suggest that IFN- γ modulates neuronal functions by inhibiting synapse formation, modulating synaptic balances and perturbing glutamate receptor clustering in the hippocampus. We have recently demonstrated that the synergistically acting cytokines IFN- γ and tumor necrosis factor alpha (TNF- α) decrease the spontaneous excitatory postsynaptic activity and alter the firing pattern in the circadian rhythm generator, the suprachiasmatic nucleus (SCN). To evaluate the long-term effects of these cytokines on SCN neuronal activity, we treated dispersed SCN cultures with IFN- γ , or a cocktail of IFN- γ and TNF- α in combination with bacterial lipopolysaccharide for 4 weeks and analyzed the effect on the spontaneous impulse and postsynaptic activity of SCN neurons. The average impulse frequency was significantly decreased and had a markedly more irregular firing pattern. Postsynaptic currents (PSCs) in the cultures were identified as GABAergic and could be blocked with GABA_A receptor antagonists. The average frequency, but not amplitude, of the PSCs was significantly decreased after treatment with IFN- γ and cocktail. Western blots demonstrated that NMDA, AMPA/kainate and GABA_A receptors were present in the cultures. These results suggest that long term treatment with IFN- γ and TNF- α induce irregular firing activity and decrease GABAergic synaptic strength in SCN neurons that could modulate the SCN output during conditions associated with increased cytokine release, such as infections and diseases that include sleep and rhythm disturbances.

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CLOCK GENES IN TORPID HAMSTERS

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Circadian rhythms are still expressed in daily torpor implying a temperature compensation of the pacemaker. Nevertheless it remains unclear, how the clock works in hypothermic states and whether torpor itself affects the circadian system. To reveal changes in the clockwork during torpor, we compared clock gene and neuropeptide expression by in situ hybridisation in the SCN and pineal gland of normothermic and torpid Djungarian hamsters (*Phodopus sungorus*). Animals from LD 8:16 were sacrificed at 8 time points throughout 24 hours. To investigate the effect of a previous torpor episode on the clock, we killed a group of normothermic hamsters one day after torpor. In normothermic animals *Per1* peaked at ZT4 whereas *Bmal1* reached maximal expression between ZT16 and ZT19. *Avp* mRNA in the SCN showed highest levels at ZT7, *Aanat* expression in the pineal gland between ZT16 and ZT22 in non-torpid animals. In torpid animals *Per1* was significantly overexpressed with the peak shifted to ZT7. *Bmal1* did not show significant changes during hypothermia. AVP expression was slightly reduced compared to normothermic animals with a plateau between ZT4 and ZT10. *Aanat* expression showed a significantly advanced onset during torpor and reached its maximum at ZT13. One day after torpor *Per1* amplitude was comparable to the control group again, whereas the peak of expression remained shifted to ZT7. *Bmal1* showed low mRNA levels with no rhythm on the day after torpor, whereas *Avp* expression was reduced by 40%. *Aanat* expression was still advanced. We conclude that although the clock seems to be impervious to temperature during torpor, torpor itself seems to affect the clock because expression of clock genes is altered following torpor.

PHOTOPERIODIC MODULATION OF SEXUAL ACTIVITY IS UNDER KiSS-1 CONTROL IN SYRIAN HAMSTER

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In seasonal mammals, like the Syrian hamster, reproduction depends upon daylength: Animals raised in short days (SD) undergo gonadal regression within 8-10 weeks, while animals kept in long days (LD) remain sexually active. This photoperiodic response is known to be mediated by the pineal gland hormone melatonin, but how it modulates sexual activity and other physiological functions remains unclear. Recently, it has been shown that the KiSS-1 gene product, Kisspeptin, plays a crucial role in the onset of puberty. Kisspeptin stimulates release of FSH/LH presumably via a direct action on GnRH neurons in the preoptic area. Neuroanatomical data support that the KiSS-1 immunoreactive fibers are found in apposition to GnRH neurons. We addressed the possible role of Kisspeptin in the photoperiodic control of sexual activity in male Syrian hamsters. We used both in-situ hybridisation and immunocytochemistry to localise KiSS-1 gene expression. We found that KiSS-1 is expressed in the mediobasal hypothalamus with immuno-reactive neurons located in a cell-clear zone located between the dorso- and ventro-medial nuclei, close to the third ventricle. A substantial number of fibers was observed in several brain regions, including the preoptic area. Importantly, *Kiss-1* gene expression is downregulated in Syrian hamsters raised in SD compared to LD. Whether this inhibition is mediated by melatonin or sexual steroids is under investigation. Finally, functional experiments showed that a chronic ICV administration of Kisspeptin to SD hamsters is able to restore full sexual activity within 4 weeks, despite a persistent inhibitory photoperiod. Together these data suggest that KiSS-1 might play a major role in the photoperiodic control of reproduction.

DIFFERENTIAL IMPAIRMENT OF FOOD SYNCHRONISATION IN *Per* MUTANT MICE

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In mammals, the master clock resides in the suprachiasmatic nuclei (SCN) of the hypothalamus. Synchronization to timed food presentation can still be achieved after SCN lesions, indicating the existence of a Food Entrainable Oscillator (FEO). The location and clockwork underlying that synchronization has yet to be identified. When food is temporally or quantitatively limited, mice show a Food Anticipatory Activity (FAA) in the hours prior to food access. Here we investigated food synchronization in wild type (WT), *Per1* knock-out (KO), *Per2* KO and *Per1/Per2* double KO mice. 96 WT, *Per1* KO, *Per2* KO, *Per1/Per2* KO mice kept under a light-dark cycle 12/12 were divided in 3 groups submitted to a calorie restriction (CR), an 8 hours access to food (TR) or ad libitum (AL) conditions, respectively. Wheel-running activity was recorded throughout the experiment. After 3 weeks of restricted feeding, the animals were transferred to constant darkness (DD) conditions and food was provided ad libitum again to assess possible phase shifts. After a few days, CR and TR WT mice showed a clear FAA and so did the *Per1* KO, while *Per2* KO and *Per1/Per2* KO did not show FAA in response to CR or TR. When released in DD/ad libitum, CR animals showed a phase shift in their locomotor activity. Temporal restriction is known to shift clock gene expression in peripheral tissues but not in the SCN whereas calorie restriction affects the SCN as well. Clock gene expression in peripheral tissues and the SCN has yet to be investigated to assess the molecular impact of CR. To sum up, the present work demonstrates that the *Per2* gene is essential in the clockwork underlying the FEO.

GENES, CELLS AND CIRCADIAN CLOCKS: DO WE KNOW AS MUCH AS WE THINK WE DO?

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Circadian rhythms represent an evolutionarily conserved adaptation to the environment that can be traced back to the earliest life forms. In animals circadian behavior can be analyzed as an integrated system - beginning with genes leading ultimately to behavioral outputs. In the last decade, the molecular mechanism of circadian clocks has been unraveled primarily by the use of phenotype-driven (forward) genetic analysis in a number of model systems. We are now in a position to consider what constitutes a clock component, whether we can establish criteria for clock components, and whether we have found most of the primary clock components? In addition, the discovery of the cellular autonomy of circadian clocks has raised a number of interesting and significant issues concerning synchronization and coherence of rhythms at the cellular level as well as circadian organization at the systems level.

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SEASONAL CHANGES IN THE CIRCADIAN ORGANIZATION OF LIZARDS: ROLE OF MELATONIN AND THE PINEAL

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Several behavioral data indicated that in the ruin lizard *Podarcis sicula* the pineal plays a central role in the circadian organization in summer and a marginal role in the other seasons of the year. Accordingly, results of endocrinological studies in constant temperature and darkness showed the existence of a robust circadian rhythm of plasma melatonin in summer (central role in the circadian system) and absence of a circadian melatonin rhythm either in spring or autumn-winter (no role in the system). However, in vitro pineal culture studies showed the existence of a circadian rhythm of melatonin in constant temperature and darkness both in summer and winter. In light-dark cycles in vitro melatonin rhythms look different in different seasons. The seasonal difference concerns melatonin peak amplitude, and not daily duration of elevated melatonin. Further in vitro culture studies carried out in summer showed that the administration of a winter photoperiod does not affect melatonin profiles. These data support the idea that information entering the pineal gland via its innervation may, in addition to the intrapineal oscillator, be involved in regulating melatonin production in the ruin lizard. Such a mechanism could explain why melatonin production in constant conditions is rhythmic in summer as well as in winter in isolated pineal glands but not in the intact animal. To test the hypothesis above we compared in vivo 24h melatonin profiles between pineal intact and pineal stalk dissected lizards both in summer and winter. Independent of the season, pineal stalk dissection induced a significant reduction of the amplitude of plasma melatonin rhythms. These data show that the intrapineal oscillator of ruin lizards is weak in all seasons, unless it is sustained by extrapineal oscillators controlling the pineal via its innervation, most likely by the suprachiasmatic nuclei.

COORDINATION OF CIRCADIAN AND CIRCANNUAL RHYTHMICITY IN MIGRATORY BIRDS - A ROLE FOR MELATONIN?

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Because most birds are diurnal, it is remarkable that a high number of species select the night for their migratory flights. This extension of the daily activity pattern to diurnal as well as nocturnal activity at particular times of year is controlled by an unidentified circannual mechanism and represents a great challenge for the circadian system regulating behaviour in these birds. Captive garden warblers exposed to different photoperiods express nocturnal activity with no linear relationship between night length and duration of nocturnal activity in variance to daytime activity that directly reflects the duration of the light phase suggesting internal constraints that limit nocturnal activity duration. Exposure to short days results in a termination of Zugunruhe whereas extreme long days result in a loss of synchronisation with the light zeitgeber although migratory activity is maintained extending into the light phase indicating the presence of differential external constraints, i.e. an adaptive range of photoperiods that allow the expression of nocturnal migratory activity and synchronisation with the light zeitgeber. Initiation of migratory behaviour in captive garden warblers kept in constant photoperiod is caused by internal desynchronisation of two behavioural components that are controlled by distinct circadian mechanisms. In these animals, melatonin production decreases inversely related to the number of animals that express migratory night activity but independent of whether the individual shows night activity or not. These data demonstrate a complex timing system controlling migratory behaviour that involves photoperiodic time measurement, certain internal and external constraints, as well as internal synchronisation of distinct behavioural components with melatonin possibly playing a role as the major coordinating signal, both in regard of daily as well as annual rhythmicity.

MELATONIN ENTRAINMENT OF CIRCANNUAL RHYTHMS

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Circannual rhythms share formal properties with circadian rhythms but are characterised by a very long intrinsic period usually 10-12 months. Two types of models (not mutually exclusive) have been proposed to account for the long-term oscillations: 1. Circannual rhythms are the product of the circadian system and by inference dependent on clock genes, and 2. Circannual rhythms are the product of a sequence of stages involving a neural network in the brain, with feedback from peripheral signals. These concepts have been investigated in hypothalamo-pituitary disconnected (HPD) Soay sheep. The HPD operation blocks the direct neuroendocrine control of the pituitary gland, but uniquely spares the photoperiod regulation of prolactin (PRL) secretion. This is thought to be because the melatonin signal that encodes photoperiod acts via the peripheral blood on melatonin-responsive cells of the pars tuberalis to control the lactotrophs. HPD sheep exposed to a change from short photoperiod (SP) to long photoperiod (LP) show a robust activation of PRL secretion from 0-12 wks (photoinduction), followed by a decline until 32-36 wks (photorefractoriness) and then spontaneous reactivation, with very consistent timing. In HPD sheep under prolonged constant LP, circannual rhythms in PRL release 'free-run' with an approximate 40-wk periodicity. There are no changes in the diurnal patterns of locomotor activity and melatonin secretion associated with the phases of the circannual PRL cycle. Changing the photoperiod, or manipulating melatonin, readily alters the timing of the long-term cycle. Parallel studies in intact sheep under prolonged LP reveal no change in circadian clock gene expression (*Per1*, *Per2*, *Cry1*, *Bmal 1*, *Rev-erba*) in the melatonin-responsive cells of the pars tuberalis. These data support the view that circannual rhythms can be generated at the level of the pituitary gland in the absence of hypothalamic input, can be reset by a change in the melatonin signal and may not depend on local tissue circadian clockwork.

MELATONIN AND SEASONAL REPRODUCTION: IMPLICATION OF CEREBROSPINAL FLUID MELATONIN IN THE INTEGRATION OF PHOTOPERIOD

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Melatonin, through the duration of its nocturnal secretion, is the primary transducer of photoperiodic information to the reproductive axis in seasonal breeders. Although this action of melatonin involves changes in the pulsatile secretion of hypothalamic GnRH, this indolamine does not act directly on GnRH neurones. In sheep it acts in the pre-mammillary area of the hypothalamus (PMH) and the signal is then brought to the GnRH neurones through interneurones. Implication of the melatonin receptor 1 (MT1) subtype in this control of GnRH secretion is suggested in sheep by the expression of *Mt1* mRNA in the PMH. Melatonin is present both in the blood and, in much higher concentrations (100 times), in the cerebrospinal fluid (CSF). Interestingly with regard to photoperiodic regulation, melatonin nocturnal duration is coded as precisely in CSF and blood and also 2-iodomelatonin binding sites in the PMH are located in the vicinity of the third ventricle. This led us to hypothesize that melatonin present in the CSF, rather than that in the blood, could transduce photoperiodic information to the reproductive axis. Melatonin is released in the CSF through a specific site in the pineal recess where protruding pinealocytes contact the CSF directly. Consistent with this specific site of release for CSF melatonin, concentrations in blood and CSF are both highly variable among individuals but are not correlated. CSF melatonin diffuses in the brain tissues and is responsible for a decreasing gradient of melatonin tissue contents from periventricular areas to more distal ones. It therefore appears to be the main source of this indolamine at least in periventricular tissues. Concerning the respective role of blood and CSF melatonin in the photoperiodic integration, melatonin carried to the brain by the blood appears to be able to mediate some effects of photoperiod on reproduction; however, CSF melatonin appears to be required to fine-tune the response both in terms of timing and amplitude.

MELATONIN, THERMOREGULATION, SKIN BLOOD FLOW AND SLEEPINESS IN HUMANS

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Many studies have indicated an important role of melatonin (MEL) in circadian regulation of human core body temperature (CBT) and alertness. The circadian rhythm in CBT is generated by a circadian pacemaker localized in the suprachiasmatic nuclei. In the evening when heat loss surpasses heat production CBT declines and sleepiness rises - in the morning the inverse occurs. The circadian rhythm of CBT and alertness are nearly in phase (low values during the night), so are MEL secretion and distal skin temperatures (DIST; high values during the night). The circadian pattern of CBT and alertness phase-lags behind MEL and DIST. These correlations seem to reflect a causal relationship, since MEL administration in the afternoon increases heat loss via distal skin regions and subsequently a reduction of CBT and alertness occurs. Furthermore, in contrast to DIST, proximal skin temperatures (PROX; e.g. thorax) follow the circadian pattern of CBT with similar circadian phase and amplitude. MEL administration in the afternoon reduces PROX in parallel to CBT. These inverse regulations of DIST and PROX can be explained by different vascular regulation of blood flow in these skin regions. Proximal skin regions contain exclusively capillaries. Skin blood flow through capillaries is a slow process and follows therefore passively the time course of CBT (conductive heat transport from core to the shell). However, the crucial factor for convective heat loss regulation is opening of arteriovenous anastomoses (AVA) in distal skin regions e.g. fingertips. Recent findings support the notion that MEL increases heat loss via amplified skin blood flow oscillations in fingertips between 0.02 and 0.15 Hz which may be induced by altered sympathetic nerve activity. Taken together, MEL seems to play an important role in nocturnal thermoregulatory changes of core and shell, which are known to have consequences for the arousal system.

PAD 6: THE “SWEET SPOT” FOR CORRECTING CIRCADIAN MISALIGNMENT BETWEEN THE ENDOGENOUS MELATONIN RHYTHM AND THE SLEEP/WAKE CYCLE

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Most totally blind people require a daily dose of melatonin in order to have their endogenous circadian pacemaker entrained to the normal phase. Normal phase is when the plasma dim light melatonin onset (DLMO), operationally defined as the 10 pg/ml threshold, is at about 21:00 in sighted individuals who habitually awaken at 07:00. The phase angle difference (PAD) between the DLMO and mid-sleep is 6 hours, rounded to the nearest integer. [PAD 6 is equivalent to a melatonin/sleep onset interval (MSI) of 2 hours and DLMO Zeitgeber Time (interval between waketime and DLMO) of 14 hours.] In order to achieve PAD 6 in most blind free-runners (BFRs) who have τ s greater than 24 hours, doses need to be taken at about 18:00; no more than 0.3 mg is needed (usually lower and sometimes much lower doses suffice). Melatonin treatment can be initiated on either the advance or the delay zone of the melatonin phase response curve (PRC), but entrainment will not happen until the entrainment point on the advance zone of the melatonin PRC eventually occurs at 18:00. Because BFRs - to a greater or lesser extent - relatively coordinate to (as yet unidentified) weak zeitgebers as described by a generalizable τ response curve [Emens et al, *Sleep* 28,A72; *J. Biol. Rhythms* 20,159 (2005)], less melatonin is needed for entrainment when taken at a later clock time; however, BFRs will have PADs shorter than 6, if melatonin is given at the normal bedtimes of about 22:00-24:00. PAD 6 appears to be the "sweet spot" for optimal mood in patients with winter depression (SAD). Before treatment, depression severity in SAD patients correlates with the extent of deviation from PAD 6. Most patients have DLMOs that are phase delayed relative to mid-sleep (PADs < 6). In all SAD patients, the more PAD deviates from 6 in either direction, the worse the depression. Particularly in delayed patients, appropriately timed low-dose melatonin treatment reduces depressive symptoms as PAD approaches the sweet spot, and overshooting PAD 6 results in a worsening of symptoms. Measurement of PAD and correcting it to 6 with either bright light or melatonin may be of benefit to people with other disorders in which the circadian misalignment component may be of clinical significance.

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ADAPTING THE HUMAN CIRCADIAN SYSTEM TO AMBIENT LIGHT ON MARS

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Known risk factors for the health and safety of astronauts include disturbed circadian rhythms and sleep loss. White light exposure of 10,000 lux (approximately 8×10^{15} photons/cm²/sec) has been used as a pre-launch countermeasure to improve circadian regulation in astronauts since 1991. The long-term goal of our current research is to determine the combination of wavelengths that most efficiently stimulates the circadian photoreceptor system and develop lighting countermeasures for long duration space exploration. Human action spectra show that short wavelength (blue) light (446-477 nm) is very potent for suppressing melatonin while the longer wavelength (575-600 nm) portion of the spectrum is relatively weak. Astronauts on missions to Mars will encounter an abundance of ambient long wavelength light above 600 nm. This portion of the spectrum was not investigated in previous human action spectra, and it is important to clarify its potency for circadian regulation before lighting countermeasures can be optimized. An initial study has compared exposure to equal photon densities (3.5×10^{14} photons/cm²/sec) of monochromatic light at 460, 630, and 700 nm plus a dark control for nighttime melatonin suppression in 8 healthy male and female subjects (mean age 23.5 ± 0.8 years) using a within-subjects design. Melatonin suppression was significantly different ($p < 0.0001$) across these wavelengths, with 630 nm and 700 nm being progressively less potent than 460 nm. Data from studies on long wavelength light can be used to engineer the ideal spectral distribution for illumination of general living quarters during space exploration.

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THE MAMMALIAN CIRCADIAN CLOCK GENE *Per2* MODULATES CELL DEATH IN RESPONSE TO OXIDATIVE STRESS

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Living in the earth's oxygenated environment forced organisms to develop strategies to cope with the damaging effects of molecular oxygen known as reactive oxygen species (ROS). Here we show that PER2, a molecular component of the mammalian circadian clock, is involved in regulating a cell's response to oxidative stress. Mouse embryonic fibroblasts (MEFs) containing a mutation in the *Per2* gene are more resistant to cytotoxic effects mediated by ROS than wild type cells which is paralleled by an altered regulation of *bcl-2* expression in *Per2* mutant MEFs. The elevated survival rate and alteration of NADH/NAD⁺ ratio in the mutant cells is reversed by introduction of the wild type *Per2* gene. Interestingly, clock synchronized cells display a time dependent sensitivity to paraquat, a ROS inducing agent. Our observations indicate that the circadian clock is involved in regulating the fate of a cell to survive or to die in response to oxidative stress, which has implications for cancer development and the aging process.

DISRUPTION OF THE RETINAL-PINEAL PATHWAY IN PATIENTS WITH CIRRHOSIS?

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Abnormalities in the 24-hour plasma melatonin profile have been reported in patients with cirrhosis and generally attributed to the effects of impaired hepatic metabolism. However, central GABAergic/glutamatergic neurotransmission is significantly altered in patients with cirrhosis and may contribute to the abnormal melatonin rhythms observed in these patients because translation of photic information from the retina to the suprachiasmatic nuclei (SCN) through the retinohypothalamic tract involves the glutamatergic/GABAergic systems. The aim of the present study was to assess the ability of light to suppress nocturnal plasma melatonin in patients with cirrhosis, as a marker of the integrity of the retinal-SCN-pineal pathway. Twelve patients with mild to moderately decompensated, alcohol-related cirrhosis (nine men, three women; mean age [range] 56 [39-76] yr) were studied. Plasma melatonin concentrations were measured under light/posture-controlled conditions over one 24-hour period and during white light exposure on the following night (91.5 mW/cm², 215 lux, 30 min, individually timed to occur on the rising phase of the subjects' endogenous melatonin rhythm). The 24-hour plasma melatonin profiles were evaluated by cosinor analysis. Light-induced melatonin suppression was calculated by comparison of the melatonin decrement at 30 min from the beginning of light administration, adjusting for baseline values. Light responses in 12 healthy individuals were recalculated from published data using the same technique. Considerable inter-subject variation was observed in the plasma melatonin profiles reflecting differences in the onset, duration and timing of the plasma melatonin peak. Light-induced suppression (mean \pm 1 SD) of plasma melatonin was significantly reduced in the patients compared to the controls (-0.099 ± 0.26 vs. -0.304 ± 0.14 ; $p = 0.026$). These data provide evidence that the disturbed melatonin rhythms observed in patients with cirrhosis may, at least in part, reflect abnormalities in the circadian response to light/dark cues, possibly as a result of cerebral neurotransmitter dysfunction.

THE RELATIONSHIP BETWEEN LIGHT EXPOSURE, MELATONIN RHYTHMS AND ABNORMAL REST-ACTIVITY TIMING IN SCHIZOPHRENIA PATIENTS: A COMPARISON WITH UNEMPLOYED CONTROLS

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Abnormal sleep patterns have been consistently reported in schizophrenia. However, relatively few studies have explored the involvement of circadian factors that modulate sleep. Schizophrenia patients are often active at night and sleep late into the day, which gave rise to the assumption that these patients stay up late because they can't hold down a job. We assessed the light exposure, rest-activity behaviour, and rhythms of urinary 6-sulphatoxymelatonin (major metabolite of melatonin) of seven schizophrenia patients with self-reported sleep abnormalities and healthy, unemployed, age and gender matched controls. We monitored each subject for six weeks in their home environment using continuous long-term activity and light recordings (Actiwatch-Light, Cambridge Neurotechnology, UK), diaries, and approximately four-hourly urine samples over 48 hours once every week. In comparison to matched employed controls, both, schizophrenia patients and unemployed controls went to bed similarly late between 0100 h and 0230 h on average, but only the schizophrenia patients slept for much longer and woke up significantly later than the employed people (sleep offset: schizophrenia: 1225 h (+/- 01:56 hrs) vs. unemployed: 0841 h (+/- 00:39 hrs)). Exposure to light remained closely associated with the time of activity rather than with the natural light-dark cycle in both, schizophrenia patients and unemployed people. However, schizophrenia patients had significantly later melatonin peak times in comparison to the unemployed people (0828 h (+/- 02:57 hrs) vs. 0512 h (+/- 01:32 hrs), respectively). These preliminary results demonstrate an involvement of abnormal circadian timing of rest-activity behaviour in schizophrenia patients with sleep problems which is associated with physiological changes, but which cannot be explained by their unemployment.

THE OLFACTORY BULB DISPLAYS INDEPENDENT CIRCADIAN OSCILLATIONS OF *Period1* IN VIVO AND IN VITRO

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Behavioral and physiological circadian rhythms in mammals are controlled by a master pacemaker in the hypothalamic suprachiasmatic nuclei (SCN). Recently, circadian oscillations of hormone secretion, clock gene expression and electrical activity have been demonstrated in explants of other brain regions. This suggests that some extra-SCN brain regions contain a functional, SCN-independent circadian clock, but in vivo evidence for intrinsic pacemaking is still lacking. We developed a novel method to image bioluminescence in vivo from the main olfactory bulbs (OB) of intact and SCN-lesioned (SCNX) *Period1::luciferase* rats. We created a window in the skull above the OB and administered luciferin locally or systemically. We then imaged bioluminescence emitted from the OB every 4 h for two consecutive days in constant darkness using an ultra-sensitive CCD camera and the In Vivo Imaging System (IVIS, Xenogen). The OB expressed circadian rhythms in situ with a reliable two-fold increase from day to night, similar to the phase, period and amplitude of ex vivo rhythms. In vivo cycling persisted for at least one month in the absence of the SCN. These results demonstrate that while the SCN may play a critical role in synchronizing locomotor activity rhythms and other circadian oscillators, it is not required for the expression of autonomous circadian rhythms in the OB.

BRAIN DERIVED NEUROTROPHIC FACTOR MODULATES GLUTAMATERGIC TRANSMISSION IN THE SUPRACHIASMATIC NUCLEUS

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The effects of light on the mammalian circadian system are conveyed by a glutamatergic projection to the suprachiasmatic nucleus (SCN) utilizing both N-methyl-D-aspartate (NMDA) and amino-methyl propionic acid/kainate (AMPA/KA) glutamate receptors. Previous studies raise the possibility that brain-derived neurotrophic factor (BDNF) and its high-affinity receptor tropomyosin-related kinase B (TrkB) may be important as modulators of this excitatory input into the SCN. In order to test this possibility, we measured rhythms in action potential discharge from SCN brain slices prepared from mice. Microdrop application of BDNF to brain slices of mice (C57 BL/6) during the night resulted in phase-shifts of the electrical activity rhythm. BDNF-induced phase shifts were dependent on glutamate receptor activation and TrkB receptor blocker (K252a) attenuated glutamate-induced phase shifts. Next, to test the hypothesis that BDNF enhances glutamatergic signaling within the SCN, patch clamp recordings of SCN neurons were performed. Brief, focal application of AMPA and NMDA in the presence of TTX, TEA and cadmium elicited inward currents in most cells in the ventrolateral SCN. Longer applications of the agonists were used for measuring the effect on the current-voltage relationship. Focal application of BDNF (100 ng/ml) had three effects on SCN neurons. First, BDNF increased the AMPA-mediated current by 62 % during the day and by 37% during the night. Second, BDNF increased NMDA-induced currents by 76% during the day and by 34% during the night. Third, evoked excitatory postsynaptic currents (EPSCs) were enhanced by 42% during the night. These effects of BDNF were blocked by the TrkB blocker (K252a). Thus our data support a functional role for BDNF as a modulator of glutamatergic transmission in SCN neurons and identify the underlying mechanisms involved.

CIRCADIAN TRANSCRIPTION AND METHYLATION OF HISTONES AT THE MOUSE *Dbp* GENE

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Mammalian circadian rhythmicity in physiology and behavior is based on transcriptional and post-translational feedback loops. In essence, the activity of the transcription factors BMAL1 and CLOCK is rhythmically counterbalanced by PERIOD and CRYPTOCHROME proteins to govern circadian gene expression. Here we show that transcriptional repression coincides with dynamic histone H3 lysine 9 methylation. Using the mouse *Dbp* gene as a model system we identified three circadian binding regions for BMAL1 and CLOCK in vivo. Although the amount of BMAL1 and CLOCK proteins was found to be nearly constant over the day in mouse liver nuclei, circadian protein:DNA complexes with different migration characteristics were resolvable in vitro. Detailed analysis revealed four different E-box motifs as important for circadian transcription and as binding sites for BMAL1:CLOCK. To gain further insight into the mode of repression we screened for histone H3 modifications. Surprisingly, transcriptional repression seemed to parallel the dynamic formation of a heterochromatin-like state all over the *mDbp* locus. In the absence of functional E-box motifs the heterochromatin-like state remained present, indicating that the conversion to euchromatin is dependent on either the binding of BMAL1 and CLOCK, and/or active transcription. These data indicate that the mammalian circadian oscillator combines rhythmic binding of BMAL1 and CLOCK and dynamic histone methylations for the regulation of target genes.

NEURONAL ACTIVATION OF RAS IN THE SUPRACHIASMATIC NUCLEUS AND PINEAL GLAND OF THE MOUSE

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RAS is an intracellular signaling protein, acting as one of the major upstream regulators of mitogen-activated protein kinase (MAPK) (ERK1/2). In order to test if RAS is endogenously regulated in the mouse brain, we investigated neuronal RAS activity over selected time-points of a day/night cycle. The possible influence of light on RAS activity was investigated in structures of the circadian system and in photosensory systems. Unexpectedly, no change of RAS activity was seen in the visual cortex during a 24h day/night period. In the suprachiasmatic nucleus (SCN), the site of the endogenous clock in mammals, RAS activity was high during the subjective day and low during nighttime. Short-term photic cues led to a rapid up-regulation of RAS, indicating a role in the photoentrainment of clock mechanisms in the SCN. The pineal gland is a neuroendocrine hand of the clock, with adrenergic signals determining the nocturnally elevated melatonin synthesis. In the mouse pineal gland, RAS/MAPK activity was elevated during night and decreased during day-time. Stimulation of explanted pineal glands with adrenergic compounds revealed that RAS activity is regulated mainly via the α -adrenergic receptor subtype and correlates with changes of MAPK phosphorylation. In a *synRas* mouse model, expressing constitutively activated V12H-RAS in neurons via the synapsin promoter, pMAPK levels in the SCN were constitutively elevated. Investigation on spontaneous locomotor activity demonstrated that *synRas* mice were resistant to light-induced phase advance shifts during subjective late night, as found in wild type litter mates (see Abstract by Jilg et al., this Meeting). We suggest that RAS is an important element in the operation of the mammalian biological clock in SCN and in the pineal gland.

ENTRAINMENT: LIFE BETWEEN CLOCKS

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The circadian clock impacts practically all functions in our body, ranging from activating genes to modifying behaviour and cognitive functions. Although circadian clocks continue to cycle in constant conditions without information about the daily changes of the environment (e.g., light and darkness, warm and cold), it has evolved to optimise the daily sequence of events within a predictably changing world. Thus, understanding how the circadian clock "entrains" to the daily environmental changes is a prerequisite to understand the function of this important biological mechanism. Entrainment of the circadian clock on the individual level is apparent by how the clock embeds itself into the 24-hour-day. In humans, this results in different 'chronotypes': some people go to sleep and are active early, others late. The distribution of chronotypes in a population forms a bell shape with the extreme early types ("larks") at one end and the extreme late types ("owls") at the other and the majority in between similar to the distribution of body height, where very short and tall people are a minority, most humans within a given population deviate more or less from the average. Chronotype is partly influenced by genetics, but also by other factors (e.g., light and age). Modern society greatly affects the circadian clock: by predominantly working indoors, we are exposed to much less light than in former times. The 24-hour society, shift work, and frequent travel over many time zones all challenge the daily programme of our bodies. While entrainment evolved to be a harmonic balance between a cyclic environment and the circadian clock, most of us now live *between* rather than *with* the external and the internal clocks challenging health and quality of life. A detailed understanding of the circadian clock and its entrainment is a prerequisite to counteract these difficulties.

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