Photic and non-photic modulation of the mammalian circadian clock

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CHAPTER 1

Introduction

Introduction

1. THE CIRCADIAN TIMING SYSTEM

Throughout the animal kingdom, species have evolved an internal time-keeping system, referred to as the 'biological clock', that allows anticipation to profound, but largely predictable, environmental day-night changes on earth. This biological clock drives 24h-rhythms in physiology and behaviour, and aligns the endogenous rhythms to the external solar day in a close temporal relationship. Being in synchrony with the environmental light-dark cycle allows the organism to cope adequately with daily changes in food availability, ambient temperature, the presence of predators, mating opportunities and/or social interactions. Additionally, the biological timing system has a major function in the regulation of seasonal rhythms in physiology. In this introductory chapter, the organizational principles of the internal time-keeping system will be discussed (section 1), and the major stimuli responsible for proper synchronization are outlined in section 2 and 3. The specific research aims that were addressed in this thesis will be introduced in section 4.

Endogenous rhythm generation in the SCN pacemaker

The circadian ('circa-dies', about a day) timing system is organized in a hierarchy of multiple circadian oscillators. The master clock resides in the suprachiasmatic nuclei (SCN) of the hypothalamus, a small bilaterally paired structure just above the optic chiasm (Klein et al., 1991; **Fig. 1**). The SCN serves as the major pacemaker that drives circadian rhythms of peripheral clocks in the body, such as in the liver, kidney, lung, heart and several brain regions (Yamazaki et al., 2000; Abe et al., 2002). In addition, the SCN drives rhythms in body temperature, hormone secretion, behavioural activity and sleep. Lesions of the SCN cause arrhythmicity of locomotor activity (Moore and Eichler, 1972; Stephan and Zucker, 1972), and transplants of fetal SCN tissue restore circadian rhythmicity in activity (Ralph et al., 1990; Silver et al., 1996), indicating its role as a major clock.

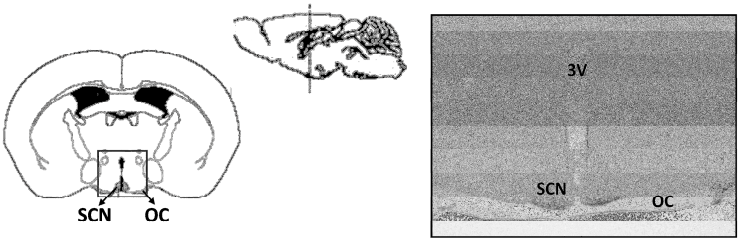

Fig. 1. Representation of the location of the SCN in the mouse brain. Left: A schematic graph of a coronal brain section. Two nuclei of the SCN are located just above the optic chiasm. The area within the square is magnified in the figure on the right. Middle: The position of the slice in the saggital brain is depicted by the vertical line. Right: Histological slice preparation of the brain. The brain section is stained with neutral red to visualise the cell nuclei. The SCN are visible as two densely stained clusters of cells embedded in the optic chiasm at each side of the third ventricle. OC = optic chiasm; SCN = suprachiasmatic nuclei; 3V = third ventricle.

Rhythm generation is established within individual SCN cells (Welsh et al., 1995) and is based on interlocked positive and negative feedback loops of clock gene transcription and their protein products. The most important clock genes are *Bmal1*, *Clock*, three types of Period genes (*Per1*, *Per2, Per3*) and two Cryptochrome genes (*cry1*, *cry2*) (for review see Reppert and Weaver, 2002; Lowrey and Takahashi, 2004). Briefly, the circadian transcription of *Per* and *Cry* genes is regulated by alternating positive actions of CLOCK/BMAL and negative actions of PER/CRY complexes. Post-translational processes such as protein phosphorylation, which is required for protein hetero-dimerization (PER/CRY) and subsequent nuclear entry of the protein complex, involve rate-limiting steps that account for a built-in time lag, resulting in a 24h clockwork.

The rhythmic expression of clock protein products leads to changes in the cell's membrane excitability, which triggers rhythms in spontaneous action potential firing frequencies (Inouye and Kawamura, 1979; Green and Gillette, 1982; Kuhlman and McMahon, 2006) and drives rhythms in humoral factors such as arginine vasopressin (AVP) and vasoactive intestinal polypeptide (VIP) secretion (Earnest and Sladek, 1986; Shinohara et al., 1995). The SCN output is established by multi-synaptic pathways to diverse relay stations, which are not yet fully identified. There is anatomical evidence for dense efferent projections to the subparaventricular zone (sPVZ), which is located just dorsal to the SCN and is thought to function in the rhythmic regulation of sleep and behavioural activity (Watts et al., 1987;

Abrahamson and Moore, 2001). Other target areas include, amongst others, the dorsomedial hypothamalus (DMH) and the preoptic area (Abrahamson and Moore, 2001). The output pathways of the SCN ultimately lead to circadian rhythms in sleep-wake cycles, physiology and endocrinology (Reppert and Weaver, 2002).

The core molecular machinery is present in every bodily cell. However, clock gene oscillations in most peripheral cells are gradually dampening after a few cycles in the absence of SCN input (Yamazaki et al., 2000), whereas the SCN oscillator is self-sustaining. Some of the peripheral ('slave') oscillators are, albeit to a lesser extent, also directly responsive to synchronizing signals other than those from the SCN, such as signals associated with feeding cycles (Yamazaki et al., 2000).

In the absence of any time cues, SCN neurons continue rhythmic firing and drive overt rhythms in behavioural activity with a period close to 24h. The circadian timing of the day-by-day onset of behavioural activity occurs with striking precision under constant conditions **(Fig. 2)**. The period of this so-called 'free-running' rhythm differs from species to species and slightly deviates from 24h. Generally taken, the period is somewhat shorter than 24h in most nocturnal animals and somewhat longer than

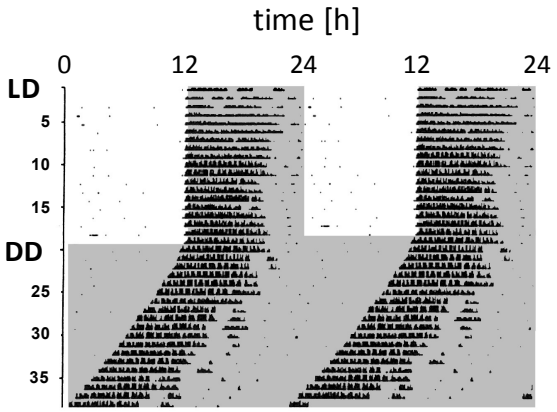

Fig. 2. Double-plotted actogram of wheel running activity of the mouse. Wheel running activity is represented by the black bars. During the first weeks, the animal was kept under LD12:12. The activity onset closely aligns to lights off, showing nocturnal entrainment to the light cycle. At day 20, the animal was released into constant darkness (DD). The animal started free running, with a period < 24h, which is apparent by behavioural activity starting slightly earlier each day. (Reprinted from Tobler et al., 1996, with permission from Nature Publishing Group)

24h in most diurnal animals. Investigations from the seventies were the first to give an estimate of the free-running period of the internal clock in humans. In these renowned experiments, volunteers were staying in a light-tight bunker refrained from any time cues for several weeks. Daily activity patterns revealed that the human clock free runs with a period close to 24.18h (Aschoff and Wever, 1962; Czeisler, 1999). However, differences between individuals exist, and likely underlie the differences in chronotype (morningness versus eveningness types of persons).

Entrainment

In order to be of functional use in everyday life, the circadian clock must be synchronized to the environmental cycle. Most organisms use the daily light-dark transitions of dawn and dusk as the principle '*Zeitgeber*' ('time provider') to adjust circadian time to environmental time. This process is termed *photo-entrainment*. 'Entrainment' implies that the rhythm of the out-read parameter is synchronized to the environmental cycle with a stable phase relationship ('phase angle') and with equal period. Apart from light, the circadian pacemaker can be reset by a variety of non-photic cues, which is described in further detail in section 3.

How does retinal illumination result in the adjustment of the core molecular clock within the SCN? The SCN receives light information from ocular photoreceptors via the retinohypothalamic tract (RHT; Moore, 1973). The light input pathway is principally mediated by glutamate (Ebling, 1996). The RHT also contains pituitary adenylyl cyclase activating peptide (PACAP; Hannibal, 2002), which is thought to modulate the glutamatergic input to the SCN (Michel et al., 2006). Different subclasses of glutamate receptors are present on SCN neurons, i.e. N-methyl D-aspartate (NMDA) receptors as well as AMPA and kainate receptors. Following light exposure, glutamate receptor stimulation leads to membrane depolarization of SCN neurons and subsequent calcium influx into the cell through voltage-dependent calcium channels. Cytosolic calcium acts as a key intracellular signal transducer in the light resetting pathway of the molecular clock. Increased cytosolic calcium levels activate a complex biochemical signalling cascade, which involves the phosphorilation of cAMP response element-binding protein (CREB), and ultimately regulates the transcription of *Per1* and *Per2* clock genes (Meijer and Schwartz, 2003). The acute light-induced *Per1* and *Per2* expression during the night seems to be the principal event for producing a phase shift of the circadian rhythm (Shigeyoshi et al., 1997; Albrecht et al., 1997; 2001; Shearman et al., 1997; Yan and Silver, 2002; Nagano et al., 2009).

SCN structural organization

While autonomous circadian rhythms in clock gene expression and electrical discharge rate are present at single SCN cell level, it cannot be excluded that rhythms in a minority of SCN cells are driven by rhythmic neighbouring cells. Nonetheless, rhythms from dissociated neurons show inter-individual variability in phase and period (Welsh et al., 1995). Thus, in order to provide a coherent rhythm output, the rhythms of the individual neurons must be synchronized. How cellular communication and coupling within the SCN is organized is subject of extensive investigation. Communication between SCN neurons and between subpopulations of neurons is thought to occur through neuronal firing and release of chemical compounds at synaptic terminals. The primary candidates of the proposed mechanisms include GABA, VIP, vasopressin, GRP and substance P. Also electrical coupling through gap junctions may be involved (Michel and Colwell, 2001).

The SCN is a heterogeneously organized population of cells, with sub-regions serving different functions. For instance, being a bi-paired structure, the left and right SCN at either side of the third ventricle can each drive circadian rhythmicity (de la Iglesia et. al., 2000; Ohta et al., 2005). Under normal conditions, however, the left and right nuclei are coupled and act in synchrony. Furthermore, an important distinction can be made between dorsal and ventral SCN regions (Van den Pol, 1980). Dorsal and ventral regions show differences in peptide expression; ventral SCN cells typically produce vasoactive intestinal peptide (VIP) or gastrin relasing peptide (GRP), whereas dorsal SCN neurons contain vasopressin (VP) or somatostatin (Antle and Silver 2005; Abrahamson and Moore, 2001; Moore, 2002). The ventral part is the light-recipient region, as most of the optic nerve fibers terminate in this area, and also receives major input from the intergeniculate nucleus (IGL) and the raphe nuclei. The dorsal region is innervated by the ventral SCN and by afferent pathways from the cortex, basal forebrain and hypothalamus. For dorsal and ventral SCN to be synchronized, interregional communication is thought to rely primarily on GABA (Albus et al., 2005). GABA is expressed throughout the SCN (Moore and Speh, 1993) and has inhibitory as well as excitatory effects on SCN neurons (Albus et al., 2005). In mice, the anatomical distinction between dorsal and ventral areas is less clearly defined than in rat or hamster, however, the functional properties of both areas seem to be still intact (Vansteensel et al., 2008).

Network properties

As stated above, circadian rhythms are cell-autonomous. Yet, many functional properties cannot be explained on the single cell level. For instance, experimental

investigations have shown that individual neurons do not code for day length. Instead, photoperiod-encoding arises at a neuronal network level (Schaap et al., 2003; VanderLeest et al., 2007; Rohling et al., 2006a; 2006b). SCN waveform responses to photoperiod can be explained by a change in the phase distribution between neurons (**Fig. 3**). The timing of neuronal activity of individual cells shows greater variation in long day photoperiods, whereas under short day lengths the neurons are more tightly in phase with each other, leading to broad and narrow peak widths, respectively, at the population level. As a whole, the SCN is capable to encode for day length. This example indicates that the interaction between cells, or between subpopulations of cells, gives rise to additional properties at a network level that are not present at the individual cell level. Thus, the output is more than the sum of parts (VanderLeest et al., 2007; Rohling et al., 2006a; 2006b).

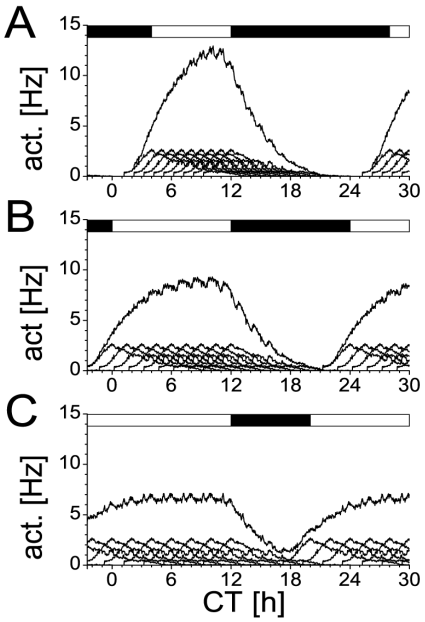

Fig. 3. Simulation of SCN multiunit activity pattern under LD 8:16 (short day), LD12:12, and LD 16:8 (long day). Each figure shows the firing pattern of 9 individual neurons, and the resultant activity pattern of the ensemble. Upper graph: when the individual neuronal activity profiles are close together, the resultant multiunit activity pattern is narrow with a high amplitude. Bottom graph: when the distribution pattern of individual neuronal activity profiles is more widely spread, the width of the resultant multiunit activity pattern is increased and has a lower amplitude. The middle graph is an intermediate of the top and bottom graph. Light dark schedules are indicated above each graph. (Reprinted from Schaap et al., 2003, with permission of National Academy of Sciences, USA)

Insight into the temporal relationship between different organizational levels within the circadian system requires experimental investigations that incorporate a combination of techniques. These comprise single- to multi-cell level measurements, including molecular recordings from SCN genetic clockwork; neuronal activity recordings from single SCN cell, from the isolated SCN network, and from the SCN as integrated part of the brain network; and recordings from functional output or overt

behaviour. Some of the well-established outread-parameters of circadian rhythmicity are described in brief below.

SCN electrical activity
Electrical activity from rodent SCN neurons can be recorded *in vitro* from slice cultures or dispersed cell cultures, which may allow single cell measurements in isolated SCN neurons ('single-unit' recordings); or from acute hypothalamic slice preparations with stationary electrodes, providing extracellular recordings of the firing activity of a population of neurons ('multi-unit' recordings; **Fig. 4**). Acute slice recordings usually last for approximately two cycles (~ 48 hours) and the SCN stays in relative isolation from afferent pathways. In addition, SCN firing activity can be recorded from microelectrodes implanted in the SCN in freely moving animals. This advanced technique of electrophysiological recordings *in vivo* provides the opportunity to perform extra-cellular long-term measurements from the SCN with all neuronal connections intact, and allows simultaneous recordings of the animal's behavioural activity rhythm.

The SCN exhibits clear circadian rhythms in spike firing frequencies, which can be mapped by counting the number of action potentials that exceed a set threshold above noise-level. Peak firing rates occur in the middle of the day and troughs occurring in the night. Thus, in nocturnal animals the SCN rhythm is in anti-phase with locomotion activity. Few other areas outside the SCN have been reported to display circadian rhythms, however, these rhythms appeared to be out-of-phase with the SCN (apart from the bed nucleus of the stria terminalis; Yamazaki et al., 1998), with peaks in firing rate during the night, in phase with the behavioural activity (Yamazaki et al., 1998; Nakamura et al., 2008).

Hormone profiles
The circadian profile of the hormone melatonin is frequently used as a marker of the circadian phase, particularly in human studies. Rhythmic melatonin secretion from the pineal gland peaks during darkness and is under the control of the SCN clock. Apart from melatonin rhythms being indirectly regulated by light, melatonin production is also acutely inhibited by light (Thapan et al., 2001). Rhythms in melatonin levels can be detected in blood plasma or saliva following repeated sampling at frequent time intervals. The timing of the 'dim light melatonin onset' (DLMO), which determines the rising phase of the melatonin profile under constant dim light condition, is a crucial parameter for determination of the melatonin phase

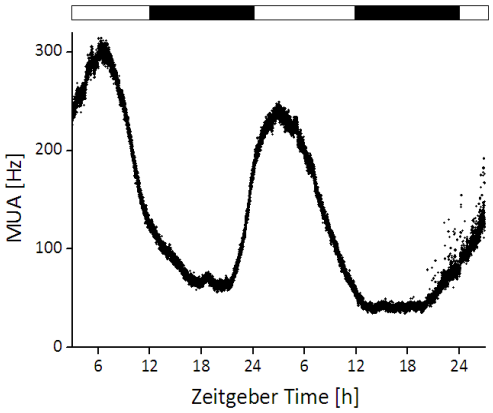

Fig. 4. Example of a 48h-record of electrical activity as measured from mouse SCN with stationary electrodes in an acutely prepared hypothalamic slice. MUA = multiunit activity. Number of action potentials that exceeded a set threshold were counted in Hz. The SCN firing rate is high during the day, and low during the night. The light-dark schedule prior to slice preparation is indicated above the graph.

position. Other rhythmically released hormones providing a tool to estimate the circadian phase include cortisol, a steroid hormone produced by the adrenal gland, and thyroid-stimulating hormone (TSH), which regulates the endocrine function of the thyroid gland. However, the latter are thought to be responsive to small modulatory effects of the sleep-wake cycle (Van Cauter, 1995).

Behavioural activity

Behavioural activity, as assessed by wheel running activity, drinking activity, or movement (locomotion) recordings, is perhaps the most extensively investigated parameter in rodent studies. In human studies, actigraphy data can be collected from wrist activity meters that rely on accelerometer measurements. Behavioural activity profiles are graphically displayed in single or double plotted actograms, representing the amount of activity as a function of circadian time, with successive cycles shown underneath each other (Albrecht and Foster, 2002; **Fig. 2**).

Sleep-wake rhythms

The timing of sleep is thought to be regulated by a circadian and a homeostatic process. There is ample evidence in support of this view, and a two-process model has been developed, which has led to testable predictions. 'Process C' refers to the circadian pacemaker which allows sleep during the rest phase of the light dark cycle, and 'process S' refers to the homeostatic process in which a need for sleep accumulates during waking. Electroencephalogram (EEG) recordings of cortical brain activity, in combination with recordings of muscle tone (electromyogram), reveal two different stages of sleep: rapid eye movement (REM) sleep and non-REM (NREM)

sleep. REM sleep is characterized by increased EEG activity but low muscle tone, as is therefore also termed 'paradoxical sleep', and is associated with bursts of rapid eye movements. Human NREM sleep can be further divided into stages 1 to 4, which correspond to successively deeper states of sleep and increasing arousal thresholds. Stages 1 to 4 are precisely defined on the basis of EEG characteristics, with low amplitude theta activity for stage 1, EEG spindles and K-complexes for stage 2 and high amplitude slow waves for stages 3 and 4. A typical nocturnal sleep pattern in humans starts with NREM sleep (stage 1 to 4 and back to 1) followed by an episode of REM sleep, and takes approximately 90 minutes for the full cycle. Subsequent cycles show increasing lengths of REM sleep as the night progresses, at the expense of NREM sleep.

Nocturnal versus diurnal animals

Most laboratory studies in the field of chronobiology have been performed in nocturnal (night-active) animals. Mainly due to a lack of a good model for diurnality, relatively few have explored the characteristics in diurnal (day-active) animals, such as the grass rat (e.g. Challet et al., 2002; Slotten et al., 2002), Octodon Degus (e.g. Lee and Labyak, 1997) and the (European) ground squirrel (e.g. Meijer et al., 1989; DeCoursey, 1997; Hut et al., 1999). Both in diurnal and in nocturnal animals, clock gene expression profiles of certain clock genes (e.g. *Per1*), as well as SCN electrical discharge rhythms, display circadian patterns that peak during the light period, and have trough levels during the dark period, **(Fig. 5)**. Importantly, the circadian rhythms of some output factors, e.g. body temperature or serotonin levels are oppositely phased in nocturnal and diurnal animals. Body temperature peaks during the day in diurnal animals, and peaks during the night in nocturnal animals. Thus, these daily rhythms are related to the animal's rest-activity cycle ("arousal-dependent cues", Challet et al., 2007). By contrast, several other rhythmically expressed factors, e.g. melatonin, are rather coupled to the light-dark cycle ("arousal-independent cues", Challet et al., 2007). Melatonin levels always peak during the dark period, irrespective of being measured in a nocturnal or diurnal animal. This implicates that the 'translation' to output behaviours is regulated downstream of the SCN, and there is a sign inversion in nocturnal versus diurnal animals, such that high levels of melatonin are equivalent to behavioural activity in nocturnal animals and to the animal's rest phase in diurnal animals **(Fig. 5)**.

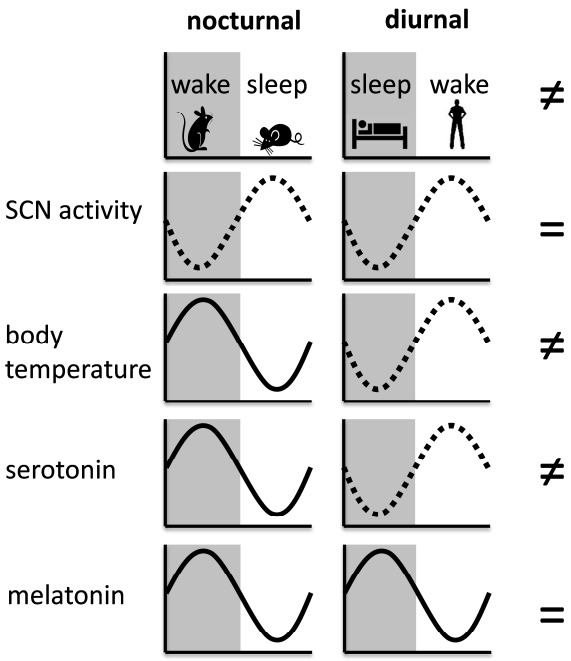

Fig. 5. Schematic representation of temporal distribution of circadian rhythms in nocturnal and diurnal animals. SCN activity is maximal during the light phase, both in diurnal and nocturnal animals. However, sleep-wake patterns, body temperature, and serotonin levels are oppositely phased in nocturnal versus diurnal species. Melatonin rhythms always peak during the dark period. Shaded areas indicate dark period (nighttime).

Circannual rhythms

Apart from driving daily rhythmicity, the SCN is involved in the regulation of seasonal rhythms such as found in reproduction, hibernation, pelage growth, and migration (see Lincoln et al., 2006). The circadian clock functions as a photoperiodic time measurement system where melatonin production from the pineal gland is thought to play a key role in seasonal rhythmicity. The SCN drives photoperiodic-dependent rhythms in melatonin, resulting in a melatonin waveform which codes for day length. Long winter nights are equivalent to broad peak widths, and short summer nights are equivalent to narrow melatonin peak widths (**Fig. 6**). A major target structure of melatonin functioning is the pars tubelaris of the pituitary. This area is involved in the seasonal regulation of several hormones, such as prolactin, luteinizing hormone (LH), follicle-stimulating hormone (FSH), and growth hormone (GH). Prolonged exposure to constant photoperiod (either long day or short day) causes photorefractoriness, which is a spontaneous reversal of the current physiological state to that of the

previous photoperiodical condition. Studies performed in Soay sheep have revealed circannual rhythms in prolactin that persist in the absence of a hypothalamo-hypophyseal connection. However, under this condition the physiological adaptation of prolactin rhythms to photoperiod was lost. It is possible that endogenous circannual oscillators exist that can cycle independently from neuronal SCN input (Lincoln et al., 2006).

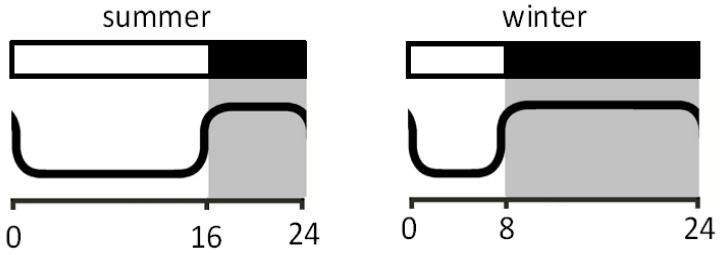

Fig. 6. Schematic representation of melatonin profiles under long photoperiod (summer time) and under short photoperiod (winter time). Shaded area indicates dark period, and LD cycle is indicated by black and white bar above the graph.

2. PHOTIC MODULATION OF THE SCN

Phase response curves

For adequate entrainment to the daily light-dark cycle, the circadian system responds differently to light at different phases of the cycle. In order to map the phase-dependent light responsiveness, light-induced phase shifts can be quantified and plotted as a function of circadian time. The general characteristics of the shape of a phase response curve (PRC) is more or less uniform for all organisms, from plant to fly to humans, from diurnal to nocturnal animals. Light pulses applied during the first part of the subjective night induce phase delays, light pulses during the second half of the night induce phase advances. During the day, the circadian timing system is largely insensitive to light, which is represented by a 'dead-zone' in the PRC **(Fig. 7)**.

Re-entrainment

Resetting of the circadian clock occurs on a daily basis to accomplish slight corrections of the phase and maintain the stable phase relationship with the environmental cycle. By contrast, larger phase shifts, as experienced in rotational shift work or transmeridian flights, may exert profound challenges to the system. By shortening the light period by 6 hours in experimental studies, or lengthening the light period by 6 hours, the animal is subjected to a phase advance or a phase delay of

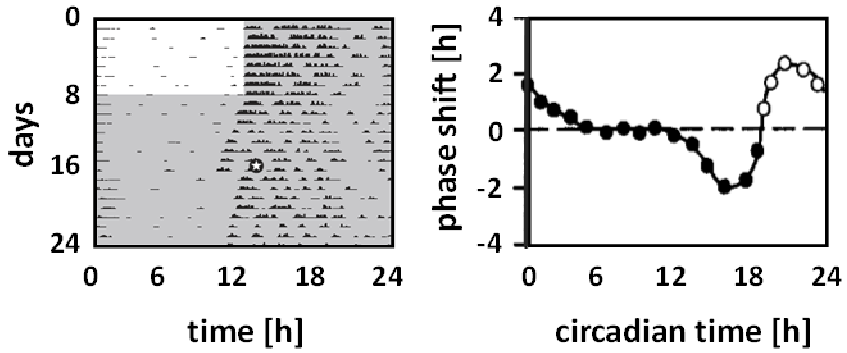

Fig. 7. Representation of a phase response curve to light. Left graph shows an actogram of wheel running activity from an animal that is entrained to LD12:12. On the 7th day in constant darkness, a light pulse is applied at circadian time (CT) 14 (beginning of the subjective night), which induces a phase delay of the animal's behavioural activity rhythm. When light-induced phase shifting responses are plotted as a function of circadian time (right graph), the PRC indicates that light induces delays during the beginning of the night, advances during the end of the night and no responses during the day. The x-axis shows circadian time, where the onset of behavioural activity in nocturnal animals is defined as circadian time 12 (CT12).

the laboratory time schedule, respectively (as in a flight from Amsterdam to New York and back), and needs to adjust its behaviour and physiology to the new time zone. Such re-entrainment paradigms (or light pulse-induced phase shift protocols) are not completed within one cycle, but develop over several transient cycles.

The mechanisms underlying the observed transients may be explained by a temporal de-synchronization of different oscillators. At the level of the SCN, different SCN components re-entrain at a different rate. Following a phase advance of the light-dark cycle, the fast phase resetting of *Per1* and *Per2* expression rhythms dissociates from the slow resetting of the *Cry1* rhythm within the SCN (Reddy et al., 2002). Further, molecular and electrophysiological studies revealed that the light-recipient ventral SCN shifts faster than the dorsal SCN (Nagano et al., 2003; Albus et al., 2005; Nakamura et al., 2005). Following a 6h delay or advance of the light dark cycle, bimodal peaks can be observed in *in vitro* SCN electrical activity recordings (Albus et al., 2005). When SCN slices were bi-sectioned, the ventral SCN was largely shifted while the dorsal SCN rhythm remained in the old phase. This effect of uncoupled areas could be mimicked by application of the GABA-A antagonist bicucculine, indicating that phase shifting of the dorsal SCN relies on the ventral SCN, and GABA is involved in the transmission of phase information from ventral to dorsal SCN (Albus et al., 2005).

A temporal de-synchronization also exists between central and various peripheral oscillators. Following a 6h-shift of the light-dark cycle, the *Per1* transcriptional rhythm from the SCN can shift rapidly, but rhythms from liver, lung and skeletal muscle each shift with different speed and resume their phase relationship after 6 days only (Yamazaki et al., 2000). The misaligned phase relationship between various peripheral oscillators is most likely the underlying cause of feelings of 'jet lag', including excessive sleepiness, early awakening, fragmented sleep, and poor performance. Crossing meridian time zones towards the east is generally experienced to cause more problems than to the west (Eastman et al., 2005). In other words, phase advances take longer and are more difficult to accomplish than phase delays. Indeed, rats showed limited capacity to shift their wheel running activity rhythms to 6h advances, but not to 6h delays of the light dark cycle (Vansteensel et al., 2003). Phase determination at different levels of organization revealed a dissociation between behavioural activity, SCN molecular activity, and SCN electrical activity (Vansteensel et al., 2003; **Fig. 8**): while the *Per1* rhythm immediately shifted to the new phase, the *in vivo* SCN electrical activity rhythm as well as the behavioural activity rhythm were not shifted to the new phase. Surprisingly, *in vitro* recordings from brain slices initially showed largely shifted rhythms of SCN electrical activity, however, when the SCN remained in situ and brain slices were prepared a few days later, the phase of the SCN appeared to be returned to the ('old') phase as measured *in vivo.* These data suggest that the SCN is retarded from shifting by inhibitory influences from 'extra-SCN' areas.

Light conditions

In the laboratory, animals can be exposed to different light conditions to untangle a variety of response features of the circadian system. In the absence of any light cues, animals express their free-running rhythms (**Fig. 2**). Therefore, the condition of constant darkness (DD) is generally used as a valuable tool to reveal the animal's intrinsic period and phase in the absence of the negative 'masking' effect of light. Masking is the phenomenon of reduced activity levels in nocturnal animals by exposure to light (reviewed in Mrosovksy, 1999). For this reason, phase response curves are usually performed under conditions of constant darkness, and steady-state phase determination of overt circadian rhythms following a shift of the light-dark cycle is most reliable when animals are released into DD.

Animals that are kept in constant light (LL) characteristically show reduced behavioural activity and lengthening of their circadian period. The brighter the light, the shorter the period, a phenomenon known as 'Aschoff's rule' (Aschoff, 1960).

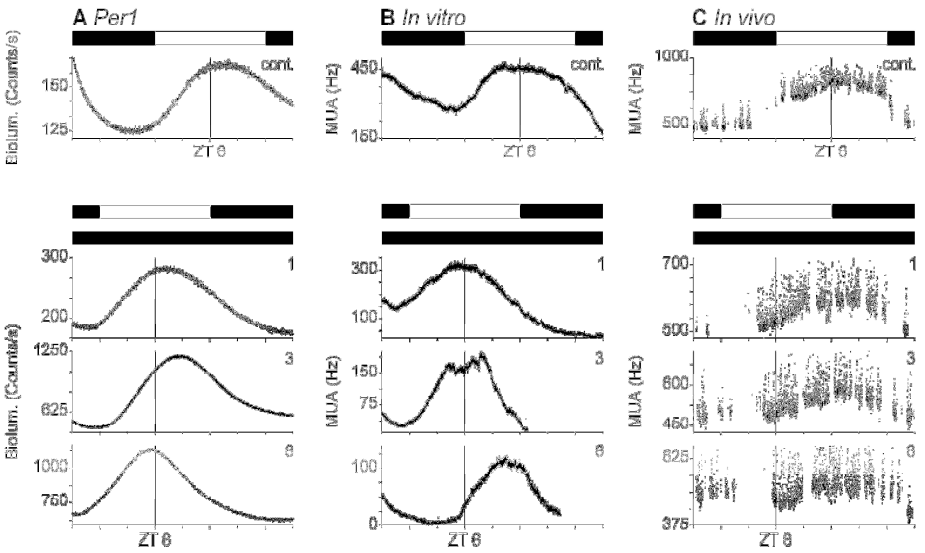

Fig. 8. Dissociation between the phase of *Per* gene expression within the SCN and SCN neuronal activity *in vitro* and *in vivo* after a phase advance of the light-dark cycle. Upper graphs of A, B and C show the rhythm profiles on the last day before the shift of the LD cycle. Bottom graphs show the rhythms on day 1, 3 and 6 in DD after the shift. Vertical lines represent Zeitgeber Time 6 of the old phase (upper graph) and new phase (bottom graphs). The light-dark schedule to which the animals were exposed prioir to the recording are indicated above the graphs. **A)** *Per1* bioluminescence rhythm is completely shifted on day 1, and remains shifted on days 3 and 6. **B)** SCN neuronal activity, recorded from a hypothalamic slice preparation, in relative isolation from the rest of the brain, is largely shifted on day 1 and 3. However, when the slice was prepared on day 6, the rhythm appeared to be returned to the old phase. **C)** Recordings of neuronal activity from the SCN *in vivo*, when brain connections are still intact, reveal that the phase of the rhythm is not shifted at all on days 1, 3, or 6. (Reprinted from Vansteensel et al., 2003, with permission from Elsevier)

Prolonged exposure to constant light conditions often leads to arrhythmicity, which is shown to result from de-synchronization of single neuron rhythms (Ohta et al., 2005). Alternatively, the activity profile can split into two distinct components, that free run out-of-phase with each other, usually about 12 hours apart. Splitting is most commonly observed in hamsters. Evidence from clock gene expression and electrical activity in hamster SCN showed that the two components reflect the oscillations of the left and right SCN that run in antiphase (de la Iglesia et al., 2000; Ohta et al., 2005).

Furthermore, circadian rhythms are also strongly influenced by day length. Photoperiod affects the duration and distribution of physiological and behavioural

activity over the cycle. In nocturnal animals, long (summer) days (LD16:8) result in narrow activity profiles during short nights, equivalent to narrow melatonin profiles. SCN electrical activity rhythms have low amplitude rhythm with large peak width. By contrast, under short (winter) days (LD8:16), behavioural activity is widely distributed over the long nights, equivalent to broad melatonin profiles. SCN electrical activity rhythms show large amplitude, narrow peak rhythms. The SCN firing rhythm is indicative for strongly coupled neurons in short days, and rather loosely coupled neurons in long days (Schaap et al., 2003; VanderLeest et al., 2007; Rohling et al., 2006a; 2006b).

Photopigments of the circadian system

What is the photoreceptive origin of entrainment by light? Light information reaches the SCN via a specialized subset of retinal ganglion cells of the inner retina (Berson et al., 2002; Hattar et al., 2002; Provencio et al., 2002; **Fig. 9.**). These cells transmit signals from rods and cones (Belenky et al., 2002; Perez-Leon et al., 2006; Ostergaard et al., 2007; Viney et al., 2007), but also have the unique property to be intrinsically photoreceptive by the expression of the photopigment melanopsin (Hattar et al., 2002; Provencio et al., 2002; Do et al., 2009). Since for long it was assumed that rods and cones were the only retinal photopigments, the last decade's discovery of melanopsin, a third class of photoreceptor, provided a breakthrough in the understanding of circadian responses. First indications of a non-rod, non-cone photoreceptor (Freedman et al., 1999; Lucas et al., 1999) led to a burst of studies exploring the melanopsin-based circadian photoresponses. Indeed, circadian-controlled aspects such as photic phase shifting (Hattar et al., 2003), light-induced suppression of pineal melatonin production (Thapan et al., 2001; Brainard et al., 2001) and the pupillary light reflex (Lucas et al., 2001) appeared to be maximally sensitive to wavelengths other than expected on the basis of scotopic (rod-mediated) or photopic (cone-mediated) responses. Action spectra derived from irradiance response curves in humans and in rodless/coneless mice, revealed maximal sensitivity at around 480 nm. This fits a template of vitamin A-based opsin, and provided evidence for melanopsin-based phototransduction.

Photopigment activation and regeneration

The rods and cones utilize an opsin protein bound with a vitamin A-based light-absorbing molecule (chromophore), called 11-*cis*-retinaldehyde. When the chromophore absorbs a photon, photoisomerization occurs, which is accompanied by a conformational change of the pigment from inactive (11-*cis*) to active (all-*trans*) state. Remarkably, the melanopsin structure shows more homology to invertebrate

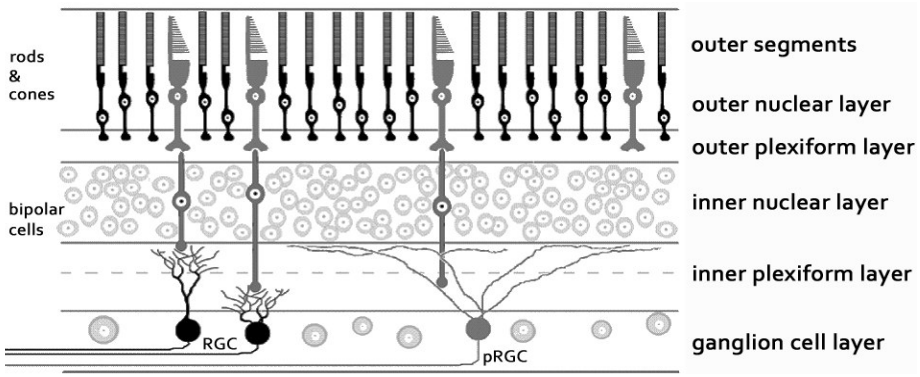

Fig. 9. Schematic representation of the mouse retina. Rods (shown in black) and cones (shown in gray) have cell bodies in the outer nuclear layer, while photoreceptive RGCs (shown in gray) have cell bodies in the ganglion cell layer. Axons of the pRGCs project to other areas in the brain. Rods and cones have connections to ON- or OFF-bipolar cells (shown in light gray) of the inner nuclear layer via synapses in the outer plexiform layer. Bipolar cells project to conventional RGCs (shown in black), as well as to pRGCs. RGCs that have dendrites in the upper part of the inner plexiform layer ('off-layer') are hyperpolarized in response to light, and RGCs having dendrites in the lower part of the inner plexiform layer ('on-layer') are depolarized. Surprisingly, dendrites of pRGCs were found to stratify in the off-layer, but pRGCs are depolarized in response to light. (modified from Berson, 2003, used with permission from Elsevier)

opsins than to vertebrate opsins (Koyanagi et al., 2005; Peirson and Foster, 2006). Further, unlike the vertebrate system, the recovery of melanopsin from the light-activated *trans*-state is thought not to rely on the 'visual retinoid cycle' (Tu et al., 2006; Doyle et al., 2006; Lucas et al., 2006), where chromophore regeneration is mediated by enzymes from the retinal pigment epithelium. Instead, melanopsin has been speculated to show bistability, which implies that it relies on a second wavelength to return to its inactive state. While some studies have favoured this theory (Koyanagi et al., 2005; Mure et al., 2007), the melanopsin photochemistry is still under debate (Mawad and Van Gelder, 2008; Melyan et al., 2005).

pRGC input and projections

The intrinsically photoreceptive retinal ganglion cells (pRGCs) constitute about 1-2% of all retinal ganglion cells (Sollars et al., 2003; Hattar et al., 2002). Melanopsin is present in cell bodies, proximal axonal parts, and dendrites of the pRGCs (Hattar et al., 2002). Unlike the conventional RGCs, the pRGCs have large dendritic fields that cover the entire retina and form a 'photoreceptive net' (Provencio et al., 2002; Hattar et al., 2002). Based on their morphology, electrophysiology and brain targets,

different subtypes of pRGCs can be distinguished (Baver et al., 2008; Ecker et al., 2010). Most abundantly present are M1 and M2 cells, but preliminary data have revealed more subclasses of pRGCs (M3, M4, M5; reviewed by Bailes and Lucas, 2010). M1 and M2 pRGCs are distinct in their stratifications: M2 cells stratify in the ON-sublamina, whereas M1 cells stratify in the OFF-sublamina. However, in remarkable contrast to conventional RGCs, even the M1 pRGCs with dendrites in the OFF-sublamina layer receive synaptic input from ON-bipolar cells (which depolarize in response to light onset; Dacey et al., 2005; Pickard et al., 2009). Therefore, pRGCs seem to receive only minor input from OFF-cone channels (which depolarize in response to light offset) (Belenky 2003; Perez-Leon et al., 2006; Dumitrescu et al., 2009).

The pRGCs project directly to the SCN via unmyelinated fibers of the RHT. pRGCs also project to other brain areas involved in non-visual processes such as the intergeniculate leaflet; the sleep regulating ventrolateral preoptic nucleus; and the olivary pretectal nucleus, involved in the pupillary light reflex (Gooley et al., 2003; Hattar et al., 2006; **Fig. 10**). More recently, first evidence has been provided for a role of melanopsin in encoding irradiance in the pathways to conventional visual areas, such as the dorsal lateral geniculate nucleus (Brown et al., 2010). With respect to the

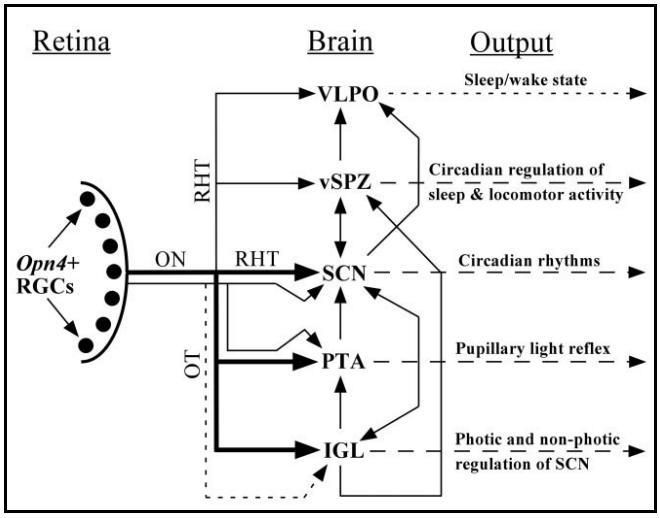

Fig. 10. Schematic diagram showing the known projections of photoreceptive RGCs. ON = optic nerve; OT = optic tract; RHT = retinohypothalamic tract; VLPO = ventral lateral preoptic area; sPVZ = subparaventricular zone; PTA = pretectal area; IGL = intergeniculate leaflet. (Reprinted from Gooley et al., 2003 with permission from the Journal of Neuroscience).

pRGC subtypes, M1 and M2 cells have been reported to signal distinct light information to various brain centers. Recent studies have suggested that the SCN primarily relies on input from M1 subtype pRGC, while both M1 and M2 subtype pRGC project to brain areas other than the SCN (Baver et al., 2008; Ecker et al., 2010; Schmidt and Kofuji, 2010).

pRGC response kinetics

Apart from the melanopsin-based photosensitivity, pRGCs also receive synaptic input from outer retinal rods and cones. Therefore, light detection by rods, cones and melanopsin seem to be integrated at the level of the pRGC. The melanopsin- and rod/cone-based photoresponses measured from pRGCs show marked differences in their response kinetics (Dacey et al., 2005; Wong et al., 2007). First, the melanopsin-based photoresponse is typically sluggish, with latency times to light onset of seconds to minutes, whereas rods and cones respond much faster (rods in the range of 100-200ms and cones in the range of 30-50ms, depending on the light intensity).

After withdrawal of the light stimulus, the melanopsin-based firing may persist for minutes, particularly after bright light pulses. Secondly, the melanopsin pigment tonically encodes the intensity of bright illumination, whereas purely rod/cone driven responses typically show transient responses to light onset and light offset. However, at intermediate to high light levels, photoresponses induced by synaptic input from rods/cones seems far more sustained in pRGCs as compared to conventional RGCs (Wong et al., 2007). Finally, the intrinsic melanopsin-based light response is less sensitive than that of rods and cones, which implies that bright light levels are required for activation of melanopsin. Due to the low melanopsin pigment density, photon capture probability is about 6log units lower as compared to rods/cones (Do et al., 2009). On the other hand, the pRGCs show spontaneous firing in the dark and in the absence of synaptic input, suggesting that the membrane potential may easily reach action potential firing threshold in the case of photon capture. Therefore, once a single photon is captured, large firing responses are obtained from melanopsin, that even surpass those of rods (Do et al., 2009).

The typical representation of transient input components driven by outer retinal photoreceptors and sustained input originating from melanopsin can be observed at several functional levels, e.g. in electrophysiological recordings from pRGCs (Berson et al., 2002; Dacey et al., 2005; Schmidt et al., 2008), in light-induced SCN responses (Drouyer et al., 2007; Mure et al., 2007), in pupillary light responses (Lucas et al.,

2001; Hattar et al., 2003; Gamlin et al., 2007; Mure et al., 2009) and in sleep induction (Altimus et al., 2008).

Relative contributions

The mammalian retina contains three classes of photopigments: rods, cones, and melanopsin. Comparing the di-chromatic system of rodents (rodent retina contains two classes of cone opsins), including mice, with the tri-chromatic system of primates (primate retina contains three classes of cone opsins), including humans, the most striking difference is the evolutionary shift of the sensitivity profile of the short wavelength cone between the blue (λ_{max} ~420-440nm; reviewed by Peirson et al., 2009) and the UV (λ_{max}~360nm; Jacobs et al., 1991). Otherwise, primates possess both middle wavelength sensitive (MWS) cones (λ_{max}~530 nm) and long wavelength sensitive (LWS) cones (λ_{max}~560 nm), while rodents only do with LWS cones (λ_{max} ~508nm; Sun et al., 1997). Rod opsin has maximal sensitivity to ~498nm (Bridges, 1959), and melanopsin (*Opn4)* is maximally responsive to ~480nm (Hattar et al., 2003; Lucas et al., 2001; **Fig. 11**). Distribution and abundance of the various photoreceptors differ between species. The number and ratio of rods and cones may be dependent on whether the animal is primarily nocturnal (rodents) or diurnal (primates).

When all pRGCs are functionally ablated (Göz et al., 2008; Hatori et al., 2008; Güler et al., 2008), photoentrainment is lost, indicating that pRGCs are the sole relay stations for photic input to the SCN. The absence of all three classes of photoreceptors (rods,cones, melanopsin) also eliminates circadian responses to light (Panda et al., 2003; Hattar et al., 2003). Circadian responses are attenuated but not absent in mice

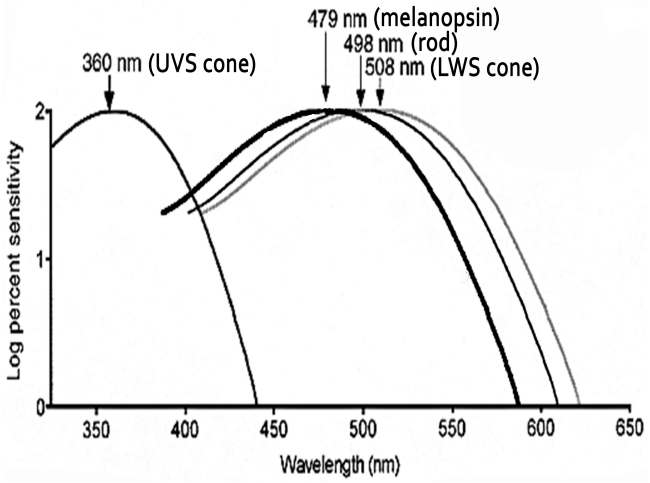

Fig. 11. Action spectra for photopigments of the mouse retina. For each opsin, the relative sensitivity is plotted as a function of wavelength, and maximal sensitivity (λ_{max}) is indicated above the plot. (Modified from Lucas et al., 2001, used with permission from Nature Publishing Group)

lacking either rods/cones (Freedman et al., 1999) or melanopsin (Panda et al., 2002; Ruby et al., 2002). The question arises what the relative contribution is of each photopigment to light detection and entrainment. The current view is that tonic irradiance detection of ambient light levels is primarily mediated by melanopsin phototransduction (Mure et al., 2007; Brown et al., 2011). Cones are considered less likely to signal sustained light information to the circadian system, due to their relatively fast adaptation physiology, which is a vital quality for pattern vision over a wide range of lighting conditions. Melanopsin signalling is important especially at bright light levels, whereas rods/cones contribute to circadian functioning at dimmer light. However, recent photoentrainment studies from several transgenic mouse lines (rodless mice, rod-only mice, red-cone knock-in mice) have revealed a contribution for rods not only at scotopic light intensities, where it uses rod bipolar pathways, but also at higher light levels, where rod signalling was demonstrated to make use of the cone circuitry (Altimus et al., 2010; Lall et al., 2010). Paradoxically, the role for cones alone in photoentrainment seems to be limited (Lall et al., 2010; Dollet et al., 2010).

Light responses in the SCN

In vivo recordings of SCN electrical activity have shown that SCN neurons are highly responsive to retinal illumination (Aggelopoulos and Meissl 2000; Drouyer et al., 2007; Mure et al., 2007; Meijer et al., 1986; 1992; 1998). The effects of light have been mimicked *in vitro* by electrical stimulation of the optic nerve, or by application of NMDA to the hypothalamic slice (De Vries et al., 1994; Ebling, 1996; VanderLeest et al., 2009). White light exposure leads to increased electrical discharge levels, which is maintained at a steady plateau for the full duration of the light pulse (**Fig. 12**). The SCN response pattern typically shows fast transient responses at lights on ('on-excitation' response) and lights off ('off-inhibition' response).

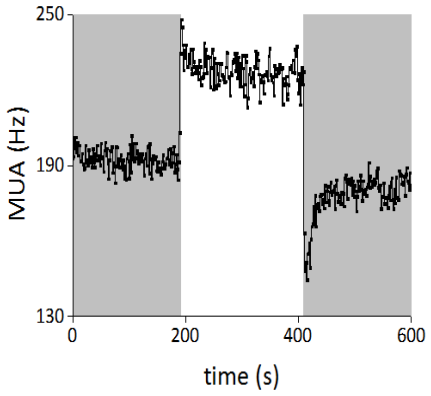

Fig. 12. SCN electrical discharge in response to a light pulse of approximately 200s. White background represents lights on, and shaded background represents lights off. The light-induced response typically shows a transient on-excitation, a sustained response during lights on, and a transient off-inhibition.

While most cells respond with increases of neuronal discharge, in a small percentage of recordings decreased activity levels have been found in response to light (Meijer et al., 1986; Meijer et al., 1998). Not all SCN cells are light responsive. Recordings from single SCN cells have revealed that light responses were present in about one third of the cells (Groos and Mason, 1980; Meijer et al., 1986). The magnitude of the light-induced SCN firing response is dependent on irradiance: the brighter the light, the larger the response, until saturation is reached, usually at about 3 log units above threshold (Meijer et al., 1986). 'Luminance coding' was first suggested by Barlow and Levick (1969) in the cat. The SCN firing rate response magnitude is also dependent on the time of the circadian cycle. Substantial excitations have been found during the night, while small or no responses were observed during the day (Meijer et al., 1998).

3. NON-PHOTIC PHASE RESETTING

While light is considered the most important Zeitgeber in mammals, a wide variety of so-called 'non-photic' factors can also reset the circadian pacemaker. For instance, behavioural activity and sleep are known to provide feedback to the circadian timing system (Mrosovsky, 1996; DeBoer et al., 2003). Increased running wheel activity during the day can alter the phase and free-running period of the circadian rhythm. Phase shifting effects have been found in response to a number of experimental treatments such as novel wheel exposure, cage changing, social interactions, restricted feeding, dark pulses and sleep deprivation (Mrosovsky 1988; Mrosovsky et al., 1996; Antle and Mistlberger, 2000; Yannielli and Harrington, 2004; Canal and Piggins, 2006). Many of the non-photic stimuli have in common that they induce behavioural activity, which suggests that the presence of behavioural activity or a state of arousal may be the critical factor for the phase shifting effects on the circadian pacemaker (Van Reeth and Turek, 1989), although unambiguous evidence is still lacking. Furthermore, phase resetting can be induced by application of several pharmacological substances, including neuropeptide Y (NPY), serotonin (5-HT) agonists, opioids (e.g. fentanyl), and short-acting benzodiazepines (e.g. triazolam, midazolam) (Albers & Ferris, 1984; Mrosovsky, 1988, 1996; Wee & Turek, 1989; Tominaga et al., 1992; Marchant & Mistlberger, 1995; Meijer et al., 2000; Vansteensel et al., 2003). These agents have been suggested to either induce behavioural activity (Van Reeth and Turek, 1989; Mrosovsky and Salmon, 1990), or they may target the neurotransmitter pathways involved in non-photic phase resetting.

Afferent pathways

Apart from retinal glutamatergic input from the RHT, the SCN receives input from two major afferent pathways, which include the serotonergic projections from the midbrain raphe nuclei, and the geniculohypothalamic tract (GHT) from the intergeniculate leaflet (IGL) of the thalamus. The latter utilizes neuropeptide Y (NPY), GABA, enkephalin and neurotensin as its neurotransmitters. These pathways are considered to be important in non-photic signalling. Lesions of the serotonergic pathway terminals within the SCN impairs the phase-shifting effects of several non-photic stimuli (Challet et al., 1997; Cutrera et al., 1994; Edgar et al., 1997; Marchant et al., 1997). Infusion of NPY antiserum onto the SCN *in vivo* blocks the phase shifts in response to novel wheel running (Biello et al., 1994). Increased behavioural activity has been reported to increase the levels of serotonin in the SCN and IGL (Dudley et al., 1998; Grossman et al., 2004), and depletion of central 5-HT levels inhibits phase resetting by arousal (Sumova et al., 1996; Marchant et al., 1997). Wheel running activity has also been reported to induce NPY release and endogenous opioids such as endorphins (Biello et al., 1994).

Phase resetting by non-photic stimuli

A number of the non-photic stimuli have been reported to suppress *Per1* and/or *Per2* expression during the day (Horikawa et al., 2000; Yokota et al., 2000; Fukuhara et al., 2001; Maywood and Mrosovsky, 2001; Maywood et al., 2002), which explains the resetting capacity at the level of the core molecular clock. Episodes of spontaneous behavioural activity lead to immediately suppressed firing activity of SCN neurons *in vivo* (Schaap and Meijer, 2001; Yamazaki et al., 1998). *In vitro* recordings of SCN electrical activity have shown that application of NPY (Liou and Albers, 1991; van den Pol et al., 1996; Cutler et al., 1998; Gribkoff et al., 1998) and 8-OH-DPAT (5-HT1A/7 receptor agonist; Prosser 1990; 2006; Shibata et al., 1992) also results in suppression and/or phase shifts of the neuronal firing frequencies.

The sensitivity of the circadian pacemaker to non-photic stimuli depends on the phase of the circadian cycle and can be described by a non-photic PRC (Rosenwasser and Dwyer, 2001; **Fig. 13**). Non-photic stimuli induce maximal phase shifts during the mid-subjective day, and smaller phase delays during the subjective night. PRC's of treatments that stimulate behavioural activity, such as novelty-induced wheel running and benzodiazepine injections, roughly resemble those of pharmacological injections of NPY or serotonin receptor agonists (Rosenwasser and Dwyer, 2001). The PRC to non-photic stimuli clearly differs from the PRC to light **(Fig. 7)**. While light pulses induce phase shifts during the subjective night, non-photic stimuli induce

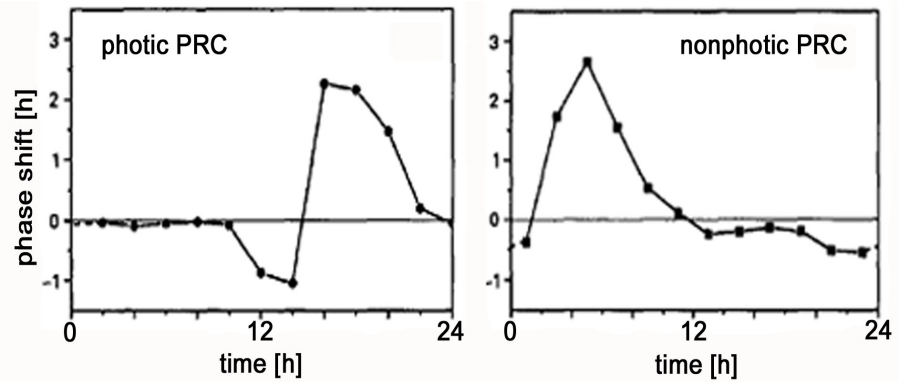

Fig. 13. Representation of a phase response curve to photic stimuli (left) and non-photic stimuli (right). The photic PRC was obtained from 1h light pulses, the non-photic PRC was obtained from 3h pulses of novelty induced wheel-running. Magnitude and direction of the phase shifts are shown on the Y-axis. Circadian time is shown on the X-axis. CT12 is defined as the time of behavioural onset of activity. While the PRC amplitudes are very similar, the timing of the phase advances, phase delays, and unresponsive regions of the PRC are opposite. (Reprinted from Mrosovsky, 1996, with permission from John Wiley & Sons)

phase shifts during the subjective day. The non-photic PRC is thus out of phase with the light pulse PRC (DeCoursey, 1964; Daan and Pittendrigh, 1976; Takahashi et al., 1984).

Photic and non-photic interactions

Photic and non-photic stimuli are capable to modulate each other's phase shifting responses. Light can block non-photic shifts during the subjective day. Phase advances induced by serotonin agonists, NPY, wheel running running, or sleep deprivation during the day can be blocked by light (Biello and Mrosovsky, 1995; Challet et al., 1998; Grossman et al., 2000; Ehlen et al., 2001; Maywood et al., 2002) or glutamate (*in vitro* SCN slice; Biello et al., 1997).

Vice versa, non-photic stimuli can block photic shifts during the subjective night. For instance, nocturnal light-induced phase shifts can be blocked by serotonin receptor agonists, NPY, GABA receptor agonists, and wheel running activity (Rea et al., 1994; Pickard et al., 1996; 1999; Weber and Rea, 1997; Gillespie et al., 1997; Weber et al., 1998; Mistlberger and Antle, 1998; Gannon et al., 2001; Lall and Biello, 2003). The serotonergic attenuation of light-induced phase shifts is established by modulation of presynaptic 5HT1B receptors and subsequent glutamate release at RHT terminals

(Selim et al., 1993; Rea et al., 1994; Pickard et al., 1996; Weber et al., 1998; Smith et al., 2001), as well as by activation of the postsynaptic 5HT7 receptor of SCN cells (Ying and Rusak, 1997). Remarkably, the counteracting effect of either NPY application or novel wheel induced running activity is present even when the non-photic stimulus is applied up to 60 minutes after the light pulse (Yannielli and Harrington, 2000; Lall and Biello, 2002). This suggests that the non-photic input interferes secondary within the light-induced signalling cascade that leads to a phase shift.

Both the IGL and the raphe nuclei receive input from the retina, and fibers from the IGL and raphe synaptically contact the RHT terminals, which provides a neuroanatomical basis for the integration of photic and non-photic information.

Finally, non-photic stimuli can potentiate light-induced shifts. When NPY and serotonergic pathways are blocked simultaneously, by using Y5 receptor antagonists (CP) and 5HT1A partial antagonists (NAN-190 and BMY), extremely large phase shifts (> 7h) can be observed in response to a light pulse in the hamster (Lall and Harrington, 2006). Both antagonists are capable to potentiate the light-induced phase shift when applied alone, however, combined application leads to shifts that are even larger than expected on the basis of the summed effects.

Role of endogenous levels of non-photic agents

Interestingly, a brief period (i.e. 2 days) of constant light markedly potentiates the phase shifting response to 8OH-DPAT (5HT1A/7 receptor agonist) in hamsters (Knoch et al., 2004; 2006; Duncan et al., 2005). The mechanism underlying this strong (type 0) phase resetting is not yet elucidated. This phenomenon seems to involve an increased sensitivity to non-photic stimuli only, since it is also seen in response to novel wheel exposure, sleep deprivation, or intra-SCN injection of NPY (Knoch et al., 2004; 2006), but not to *in vivo* application of NMDA (Landry and Mistlberger, 2005). A putative explanation could be that constant light reduces the endogenous levels of serotonin and the amount of locomotor activity, leading to increased sensitivity to serotonin agonists. Indeed, the endogenous levels of non-photic agents, serotonin in particular, have been reported to be of critical importance for the resultant phase shifting response. Phase shifts can be enhanced by depletion of endogenous 5HT levels (e.g. by pretreatment with either a 5HT-synthesis inhibitor or a 5HT7 antagonist), and, by contrast, pretreatment with serotonin agonist with subsequent high 5HT levels leads to a decreased sensitivity to serotonin-induced phase shifts (Prosser et al., 2006).

4. RESEARCH OUTLINE

It is well established that the regulation of 24h rhythms in physiology and behavior is regulated by a central pacemaker that resides in the suprachiasmatic nucleus of the hypothalamus. The various endogenously timed bodily rhythms need to operate in close harmony with each other and with the environmental day-night cycle. The temporal organization is under the influence of external time cues, which allow the circadian timing system to adjust to daily and seasonal changes brought about by the rotation of the earth. The capacity of the endogenous body clock to cycle autonomously, to being receptive to environmental signals, and to encounter flexibility as well as limitations in phase adjustment, raise intriguing research questions about the working mechanism of this timing system. This thesis evaluates the correlation between the SCN and behaviour, and particularly aims to investigate the modulatory role of external factors involved in re-entrainment. It focuses on the speed at which phase adjustments are established at different levels of circadian organization, and examines how the SCN pacemaker responds to different input signals of photic and non-photic origin to keep synchrony with the external world.

Chapter 2 focuses on the regulation of the behavioural activity rhythm by the SCN, and investigates how the onset and offset of behavioural activity correlates with the SCN electrical activity waveform pattern.

Chapter 3 explores the phenomenon of the circadian timing system adapting only slowly to a phase shift of the environmental light-dark cycle. Adjustments to advanced light-dark cycles (corresponding to westbound flights) are particularly hard to establish and require several days more than delays (corresponding to eastbound flights). From previous studies in our lab we learned that this slow adaptation response is not due to a slow adapting SCN itself, but rather involves influences from extra-SCN areas. The key question is where this 'inertia' has its origin. Here we use a recently generated mutant mouse model that is known for its increased capability of synaptic communication, due to altered functioning of a subtype of presynaptic calcium channels. This mutation ultimately leads to increased neurotransmitter release at the nerve endings. We hypothesize that the phase shifting kinetics are changed in this mouse model. To this end, we measure circadian rhythmicity at different levels of organization (behavioural activity, sleep, SCN in brain slices, SCN in intact animals) to study the underlying mechanism.

The experiments reported in **chapter 4** investigate how the murine SCN pacemaker is influenced by monochromatic ultraviolet light. Photic signals are of major importance for synchronization of the mammalian circadian timing system to the environment. In contrast to humans, the short wavelength sensitive cone pigment of the mouse retina has a high sensitivity to ultraviolet light. Functional participation of cone pigments in circadian regulation has been considered rather unlikely, since the fast-adapting physiology of cones in response to light seems unsuitable for sustained irradiance detection tasks. Instead, irradiance signalling has been largely ascribed to melanopsin-based phototransduction. **Chapter 4** explores the role of UV light sensitive cones within the circadian system by taking advantage of different retinal mutants and recording the impact of UV light on SCN electrical activity patterns, behavioural activity patterns and sleep.

While light is the most important Zeitgeber for the mammalian circadian system, the SCN pacemaker is also responsive to non-photic input. For instance, application of opioids can affect the phase regulation of the SCN clock. Endogenous opioids are also present in the afferent connections from the raphe nuclei, one of the major pathways involved in mediating non-photic phase shifts. In **chapter 5** we evaluate the phase shifting actions of opioids on behavioural activity patterns. We assess how opioids exert their modulatory action within the hamster SCN, both at the level of SCN electrical discharge and SCN clock gene expression and try to elucidate the type of receptor responsible for the circadian and phase shifting effects. Finally, we investigate how opioid- and light-induced phase shifts interact.

The circadian system is also affected by behavioural activity, which is known to induce phase shifts during the day. Thus, apart from the SCN driving circadian rhythms in overt behaviour, the behavioural activity has a reciprocal modulatory role on the SCN. Spontaneous behavioural activity leads to acute suppression of the SCN firing rate. These effects are superimposed on the SCN electrical rhythm and is the topic of **chapter 6**. The relation between neuronal suppression and type and intensity of the behaviour is analysed and the functional implications for the SCN rhythm amplitude is evaluated. Moreover, by use of mild behavioural manipulations we examine whether there is a differential neuronal SCN response to triggered versus spontaneous behavioural activity.

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