Photic and non-photic modulation of the mammalian circadian clock

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CHAPTER 7

Discussion, conclusions and implications

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A fundamental property of the circadian system is that it entrains to the environmental day-night and seasonal cycle. The master pacemaker, which resides in the suprachiasmatic nucleus of the hypothalamus, is a self-sustained oscillator and is susceptible to resetting time cues in order to remain in synchrony with the environment. A complete understanding of entrainment in a natural environment will involve the identification of the significant time cue parameters and their relative contributions.

Relation between SCN neuronal activity and behavioural activity

Overt behavioural activity patterns are markedly affected by changes in light conditions, such as changes in day length (**chapter 2**), a shift of the light-dark cycle (**chapter 3**), or brief light pulses (**chapter 3 and 4**). In nocturnal animals, exposure to long photoperiod leads to short durations of nocturnal activity, while short photoperiod leads to long durations of activity. How is this altered activity distribution in response to the environment established and what is the role of the SCN? How do SCN neuronal activity and behavioural activity correlate? Previous studies from our lab have shown that photoperiodic encoding occurs at the level of the SCN (VanderLeest et al., 2007). The SCN waveform, as coherently signalled from the population of cells, shows profound changes in peak width under short and long photoperiod. In this thesis it is reported that, under all light conditions tested (LD8:16, LD12:12, LD16:8), the circadian rhythm in behavioural activity corresponds with the SCN neuronal activity level (**chapter 2**). The effects of SCN activity on the timing of activity (α) and rest (ρ) can be described by a probability function, in which the occurrence of behavioural transitions is predicted by the half maximum level of the SCN rhythm amplitude. While the SCN waveform changes under short and long photoperiod, the around-50% level acts as a threshold that determines the onset and offset of behavioural activity under each photoperiodic condition. The SCN electrical activity seems to be more tightly coupled to the behavioural onset than to the

behavioural offset, which reflects the differences in steepness of the declining and rising slope, respectively, and is consistent with the common observation that onsets of behavioural activity in nocturnal rodents are more precise and acute as compared to offsets (Pittendrigh and Daan, 1976). In view of the results from **chapter 6**, one could argue that the declining slope may be further accelerated by the presence of behavioural activity, which is shown to have acute feedback effects on the clock. In conclusion, the results from **chapter 2** show that the circadian rhythm of behavioural activity is under control of specific levels of SCN neuronal activity. Although the pathways, factors, or relay stations involved in the SCN-driven regulation of circadian rhythms in overt behaviour are not yet unravelled (Nakamura et al., 2008), the SCN electrical activity can be considered to be a first step in the output of the clock.

Photic and non-photic modulation: research questions

Apart from the SCN neuronal activity acting as the driving force in the regulation of behavioural activity, the SCN is subject to modulation by several photic and non-photic cues. Studies presented in this thesis aimed to contribute to the understanding of the mechanisms underlying entrainment to environmental day-night and seasonal cycles. How does the SCN respond to several of the modulating factors? More specifically, how does the SCN respond to a shift of the light dark-cycle (**chapter 3**)?; how does the SCN respond to UV-light (**chapter 4**)?; how does the SCN respond to opioid application (**chapter 5**)?; and how does the SCN respond to behavioural activity (**chapter 6**)? The experiments performed to cover these issues often included a combination of techniques (e.g. clock gene expression, *in vivo* and *in vitro* SCN electrical activity rhythms, sleep patterns and behavioural activity rhythms), as to unravel the response of the circadian timing system at different levels of organization. In the next sections, the results from this thesis will be discussed in the broad context of the modulating actions of external stimuli contributing to circadian entrainment under natural conditions.

Re-entrainment to a shift of the light-dark cycle.

Generally, re-entrainment to advanced light-dark cycles, as following transmeridian flights to the west, takes much longer to accomplish than adjustment to delayed light-dark cycles (eastward flights). In the laboratory, such a 'jet-lag' protocol can be simulated by exposing animals to a shift of the light dark cycle. When animals are released into constant darkness following exposure to one or more shifted cycles, the applied experimental protocol provides the opportunity to determine the intrinsic phase of the behavioural activity rhythm. Experimental investigations described in **chapter 3** reveal that wheel running rhythms of mice show only minor shifts in

response to a 6h phase advance of the light-dark cycle, while immediate large shifts were observed following a 6h delay of the light-dark cycle (**chapter 3;** Vansteensel et al., 2005). When animals were exposed to one or more additional shifted cycles before their release into DD, as explored in a separate experimental series (van Oosterhout, unpublished results), it can be observed that re-entrainment of wheel running rhythms to a 6h advance of the light-dark cycle occurs only gradually, with a maximum of approximately 2-3 h per cycle. The rhythm needs at least 4 cycles to regain its phase relationship with the environment (**Fig. 1**; Reddy et al., 2002). Apparently, the circadian system shows inertia to shift rapidly in response to phase advances, but not delays, of the light dark cycle. It could be argued that it is more difficult to speed-up, instead of slowing down, a certain process. Nonetheless, the origin of the inertia is unknown yet, and the search for the underlying cause was the main issue of **chapter 3**.

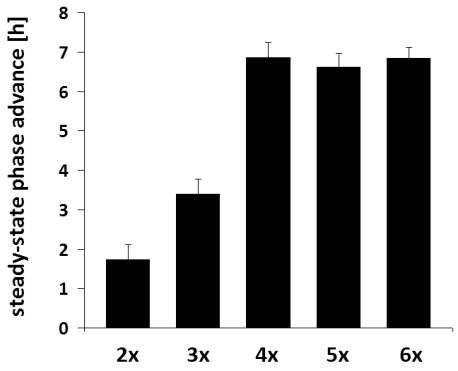

Fig. 1. Steady-state phase advance of wheel running activity rhythms of mice following a 6h advance of the light-dark cycle, as a function of the number of cycles that the animal was exposed to the new light-dark cycle. After the given number of cycles, represented on the X-axis, the animals were released into DD to determine the intrinsic phase of the behavioural activity pattern.

The inertia of the circadian system is striking, as we found that the neuronal activity rhythm of the mouse SCN (**chapter 3**), like the rat SCN (VanSteensel et al., 2005), has the capacity to shift rapidly when recorded in isolation from the neuronal network level. Therefore, the SCN seems to be retarded from phase shifting by its connections with the central nervous system. The data presented in **chapter 3** show that the inhibitory effects exerted by extra-SCN areas on SCN phase shifting is mediated by synaptic signalling through presynaptic $Ca_V2.1$ calcium channels. The results were obtained from a comprehensive study on the phase resetting kinetics in a migraine mouse model. These mice carry a gain-of-function mutation in the $Ca_V2.1$ calcium channel gene. Apart from the pathophysiological interest for this mutation, being causal to familial hemiplegic migraine in humans (see page 178-179), this mouse

model is of chronobiological interest because of its apparent increased neurotransmitter release into the synaptic clefts. Remarkably, in contrast to the minor responses of wildtype mice, the altered neuronal signalling within the migraine mouse model leads to immediate large phase advances of overt behavioural patterns. This could be measured from both wheel running activity rhythms and sleep-wake rhythms. Importantly, the SCN electrical activity rhythm as measured *in vivo* was also largely shifted in the migraine mice. Thus, the behavioural activity rhythm reflects the phase of the *in vivo* SCN electrical discharge rhythm both in wildtype mice and in the migraine mouse model. From these results we conclude that the physiological retardation from 'extra-SCN areas', that prevents the SCN from shifting in wildtype mice, is substantially reduced in the migraine mouse model. Although future research is required to elucidate the anatomical basis of the involved pathways, we now know that the inhibitory effects are established through $Ca_V2.1$ calcium channel mediated pathways.

Meanwhile, the question has arisen whether it is preferable to be equipped with a biological timing system that shifts immediately to acute large environmental changes. Instead, it may be regarded rather beneficial that the system is protected from rapid phase shifting by the presence of neuronal connections that attenuate the shifting response. Under natural conditions, resetting of the circadian clock occurs on a daily basis to accomplish slight corrections of the phase. The circadian system should provide flexibility to entrain within the range of natural occurring changes of environmental conditions. Large shifts of the light-dark cycle are unnatural and exceptionally challenging to the circadian system for resuming internal phase relationships between central and peripheral oscillators. This is also exemplified by a study on aged animals, revealing that repeated exposure to large phase advances, but not delays, lead to increased mortality rates (Davidson et al., 2006). As such, the findings described in **chapter 3** underscore the significance of functioning of the SCN as an element of a neuronal network, as the network organization will lead to greater stability of the circadian system.

The circadian response to brief light pulses

The construction of phase response curves to brief light pulses is a well-established empirical way to estimate the light responsiveness of the circadian system at a specific phase of the cycle. Qualitatively, the PRC to light pulses is similar for all species. Quantitatively, the shape of the PRC is species-specific, and within a certain species the magnitude and direction of the phase shift, and thus the shape of the PRC, is determined by the properties of the light pulse: intensity, duration, and

spectral composition. The advance portion of the mouse PRC to saturating UV-light pulses was found to be much smaller as compared to the phase delay portion (**chapter 4**). This asymmetrical shaped PRC is in accordance with previous publications on mouse PRC to white light (Pittendrigh and Daan, 1976; Sharma, 1998; Comas et al., 2006).

The circadian system is known to process light pulses on the basis of 'photic integration', with phase responses proportional to the energy of the stimulus. The system responds to light duration and light irradiance following a log-linear relationship, which illustrates that the light-triggered responses reach a maximum and no further increments in phase shift can be induced (Nelson and Takahasi, 1991; Meijer et al., 1992). Paradoxically, while full re-entrainment paradigms often have limited phase shifting effect, as described above, relatively large phase shifts can be produced by appropriately timed exposure to a single brief light pulse. For instance, a 15-min light pulse of monochromatic UV light induces a shift of >1 hour (**chapter 4**), and numerous previous studies have reported such large phase shifts by using light pulses of similar or even shorter durations (see Nelson and Takahashi, 1991). The reason for this discrepancy is still under debate. One explanation could be that a shift to a short light pulse results from triggering a subset of neurons being in equal phases, leading to a coherently induced large phase shift, while extended light exposure subsequently affects different subpopulations of cells at different phases, producing a less synchronized output and reduced phase shift magnitudes.

In addition, light-dark cycles as in the re-entrainment protocol seem to exert a stabilizing effect of the phase of the SCN pacemaker via extra SCN-areas (**chapter 3**; Vansteensel et al., 2005). Under constant darkness, which is the background condition of light pulse protocols, this stabilizing effect may be reduced, since the phase shifting effect of a single light pulse is enhanced following an increasing number of days in darkness (Refinetti et al., 2007).

SCN response to UV light

White light is known to exert direct effects on the firing pattern of SCN neurons. The response is excitatory and persists for the full duration of the light exposure. In **chapter 4**, we addressed the question how the SCN 'perceives' incoming UV light information. Since the discovery of melanopsin, the property of tonic firing is largely ascribed to the functioning of melanopsin. As the cone system is generally known to be more prone to light adaptation, the cones were considered less likely candidates for steady irradiance coding. In **chapter 4** we show that all response characteristics to

white light, including the sustained firing pattern, can be reproduced by monochromatic UV light of 365nm, which was aimed to maximally stimulate the UVS cone. This wavelength was chosen as it is fairly outside the maximal spectral sensitivity range of the other known mouse retinal photopigments (rods, MW cones, melanopsin), thereby providing the opportunity to elucidate the responses principally mediated by UVS cone opsins. To ultimately exclude the involvement of melanopsin in UV light-induced irradiance detection, the SCN neuronal response to UV light was also tested in melanopsin-deficient mice. Indeed, the response characteristics were unaltered, indicating that sustained irradiance coding can occur independently of melanopsin.

The contribution of cones to the UV light-induced SCN response is demonstrated by the very short response latency (30-40ms), which deviates from rod-mediated responses (Dacey et al., 2005; Wong et al., 2007). This implicates that at least the transient on-excitation response component is driven by cones. Interestingly, the magnitude of the on-excitation response, like the magnitude of the tonic response, was found to be dependent on irradiance and on circadian time. The functional significance of this cone-driven phasic component was recently evidenced by Lall and colleagues (2010) in a red-cone knockin mouse model. They tested the phase shifting response to a series of brief, long-wavelength, light pulses (discontinuous light pulse) and compared the outcome to the response to a longer duration (continuous) pulse with equivalent numbers of photons. Indeed, the phase shifting response was significantly enhanced in the discontinuous protocol, indicating the role of cone input to the circadian clock.

The question remains where the property of sustained signalling has arisen. Conventional RGCs were found to respond exclusively with transients to cone-mediated input (Wong et al., 2007). In pRGCs, by contrast, synaptic input from rod and/or cone pathways can trigger sustained responses under intermediate to bright light conditions. The sustained response at the level of the SCN is likely to reflect a cone-mediated light response at the level of the pRGC. This is evidenced by a recent report by Schmidt and Kofuji (2010), which shows sustained responses within the pRGC even in the absence of melanopsin. However, the latter seems to be true particularly for the M2 cells, but not the M1 cells, while the SCN has been suggested to rely primarily on the M1 cell input. This ambiguity stresses the need for clarification of light-evoked response characteristics at the level of the SCN. The current findings open up new areas for future research with respect to the retinal circuitry involved in circadian photoentrainment.

The most parsimonious explanation for the observed UV-light induced responses is the activation of UVS cones. Although cones are prone to adaptation, upon light activation their membrane potential hyperpolarizes to a peak, and then drops over time to a persistent steady-state level around half the peak polarization (Burkhardt, 1994; Normann and Perlman, 1979). Previous studies have shown that circadian phase shifting responses to UV light are unaffected in mice lacking functional LWS cones (Dollet et al., 2010). Likewise, retinal degenerated (*rd/rd*) mice, in which all retinal rods are lost, showed unaltered phase shifts in response to UV light exposure (Provencio and Foster, 1995). However, alternative mechanisms for UV photosensitivity have been proposed, including β-band absorption by opsin/vitamin A photopigments (e.g. Von Schantz et al., 1997). This explanation is now highly unlikely in view of our results obtained under saturating, long-wavelength, background illumination. These results show that UV light can still evoke persistent SCN neuronal responses under light conditions where rod, and possibly LWS cone, activity is suppressed.

Thus far, only few studies have examined the contribution of the cone system to circadian photoentrainment. These studies merely focused on LWS cones, and the artificial light sources used were generally lacking UV emission. Photoentrainment studies have suggested an important role for rod, but apparently not cone, phototransduction (Altimus et al., 2010; Lall et al., 2010). However, neither study examined the role of UVS cones in particular. Our study is the first to study the role of UVS cones in the circadian and sleep systems. Whether the capability to induce sustained responses at the level of the SCN is a property uniquely to UVS cone pathway, or may also be valid for the other class of cones (LWS cones), remains to be elucidated. In primates, the short wavelength-sensitive cones (SWS) and middle/long wavelength sensitive (MWS/LWS) cones have distinct input to the pRGCs, i.e. SWS cone mediated responses have depolarizing effects on the pRGC and MWS/LWS cone mediated responses are hyperpolarizing (Dacey et al., 2005). This phenomenon has not (yet) been observed in rodents.

The function of UVS cones

UVS pigments are co-expressed with LWS pigments in a large fraction of murine cones (Rohlich et al., 1994; Lyubarsky et al., 1999; Applebury et al., 2000; Jacobs et al 2004; Nikonov et al., 2006). The retinal distribution of cone pigments shows a dorso-ventral gradient, with short wavelength sensitive cone pigments being more dominantly present in the ventral half of the retina (Rohlich et al., 1994; Szel et al., 1992, 1996, Nikonov et al., 2006). This co-expression seems not ideal for the function

of contrast vision, however, the spatial arrangement ('looking to the sky') seems optimal for the perception of UV-light from solar radiation. UVS cones may play an important role in twilight detection. The spectral composition of sunlight changes during the day and shows a significant increase in the ratio of ultraviolet to longer wavelength light during dawn and dusk (Hut et al., 2000, Hisdal et al 2005). The contribution of multiple photopigments, including UVS cones and other opponent channels, may be of use in the detection of the dynamic pattern of light at dawn and dusk. In this way, UV-sensitivity could contribute to photic entrainment mechanisms in nocturnal animals that operate at the early dusk and late dawn.

While we found intensity-dependent UV light responses that covered at least 3 log scales under dark conditions, indicating the capacity to encode for irradiance, we also found persistent responses under light conditions. The cone system is known to have greater sensitivity to light as compared to melanopsin, and irradiance detection tasks in addition to melanopsin, or even rods, are not fully understood yet. In view of the current results it is conceivable that the cone photopigments serve a function in irradiance signaling under a large variety of ambient light levels. This may be helpful in adaptation and fine-tuning under changing light conditions, such as in winter time when the day light is less intense, and nights are long; or during twilight, when light intensities gradually change.

Collectively, the contribution of multiple photopigments, including the UVS cones, may aid to broaden the spectral range available for photon capture, while the capacity to code for the irradiance and duration of the environmental light seems functionally retained. The different photopigments likely function at different spectral, irradiance and time sensitivity windows. By the use of different overlapping sensory channels precise entrainment can be established.

Light response in other brain areas

From additional experimental investigations (van Oosterhout and Houben, *unpublished results*), beyond the scope of the specific research questions described in this thesis, it became clear that electrophysiological activity from some brain areas other than the SCN revealed circadian oscillations. These rhythms were always in anti-phase with the rhythm of the SCN, peaking during the night. The occurrence of reversed rhythms is in agreement with recordings from literature, from the subparaventricular zone (sPVZ), the nucleus accumbens, and the ventromedial hypothalamic nucleus, amongst others (Yamazaki et al., 1998; Nakamura et al., 2008; Meijer et al., 1998). In our recordings from the sPVZ, we tested the response to light.

Exposure to a stimulus of white light (as well as UV light) resulted in a decrease of the firing activity level, which opposed the response obtained from the SCN (**Fig. 2;** Kubota et al., 1981). In one recording we have been able to measure the light-induced response from a small sub-population of cells (average firing frequency is around 5Hz).

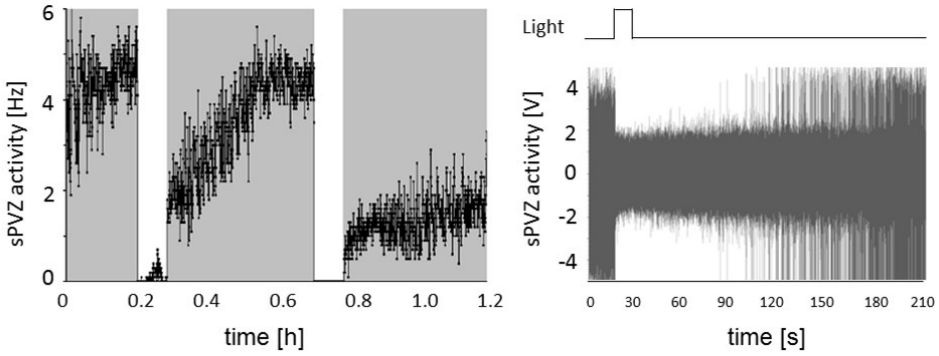

Fig 2. **Electrical activity from the sPVZ. A)** Subpopulation activity in response to a 5-min light pulses (white background = lights on). The Y axis represents the number of action potentials counted above a set threshold. **B)** Trace of electrical activity recorded from the sPVZ ("raw data"). The trace shows a complete silencing of neuronal firing as soon as the 10s-light pulse was applied. After stimulus withdrawal, firing frequency is slowly recovering.

Following light onset, the neurons acutely ceased their action potential firing (**Fig. 2**). Following lights off, the population remained silent for approximately 1 min and then started firing with increasing frequency until baseline activity levels were reached again. The reversed sign of the light response is consistent with previous electrophysiology recordings from the sPVZ (Nakamura et al., 2008). The light response offset kinetics differ from those measured in the SCN (see **chapter 4**), and also from those previously measured outside the SCN (Meijer et al., 1998). The data suggest that the SCN and the sPVZ area are coupled, and either the fibers from the retinohypothalamic tract project directly to this area, or the SCN has mediated these light responses.

Non-photic stimuli: opioids

Opioids act as non-photic stimuli that can induce phase shifts in behavioural activity rhythms. Endogeneous opioids (endorphins, enkephalins) are present in the projections from the IGL to the SCN. In **chapter 4** we tested the working mechanism

of the opioid agonist fentanyl in the hamster circadian system. Fentanyl was found to have acute inhibiting effects on the SCN electrical activity when applied to hypothalamic slices. We found that fentanyl exerts its action through true opioid receptors, since the opioid receptor antagonist was able to block the fentanyl-induced phase shifts. Several opioid receptors have been reported to be present in the SCN. While phase shifts in response to photic or non-photic stimuli has often been associated with altered levels of *Per1* and/or *Per2* clock gene expression in previous studies (Maywood et al., 1999; Horikawa et al., 2000), fentanyl was not significantly effective in suppression of *Per1* levels, although the effect on *Per2* cannot be excluded.

The results are of special interest in view of the previously reported increased production of endogenous opioids such as endorphins in response to vigorous behavioural activity. This implicates that exercise can activate the same SCN-innervating pathways as opioids.

Non-photic stimuli: behavioural activity

Interestingly, a reciprocal interaction exists between SCN neuronal activity and behavioural activity (**chapter 2 and 6**). The data presented in this thesis show that spontaneous behavioural activity has acute inhibitory effects on SCN firing rate, and quantitative analysis reveals that the effect on neuronal discharge levels is dependent on type and intensity of the behaviour. While low-intensity behaviours such as grooming or eating are effectively suppressing the neuronal activity, strongest modulation is exerted by vigorous behaviour, particularly locomotion activity. The activity-induced suppression is superimposed on the circadian rhythm, and the suppression magnitude can be (almost) as large during the night as during the day. Importantly, when the animal is behaviourally active, the SCN firing rate seems to 'switch' to a lower state of activity, that still shows circadian rhythmicity.

From the current analyses, we cannot exclude the presence of ultradian components underlying the occurrence of bouts of activity. Previous studies from hamster and rat have reported ultradian rhythms in electrical activity and behaviour with periods of 14 min, 80min and up to 4 hours (Meijer et al., 1997; Yamazaki et al., 1998). The origin and mechanism of ultradian rhythmicity are not clear yet. Data from the common vole have suggested that ultradian rhythms are not likely to arise from the SCN, but rather orginate from brain centers outside the SCN, e.g. the arcuate nucleus (Gerkema et al., 1990; Van der Veen et al., 2006).

In nature, several reasons exist for an animal to show motivated behaviour, e.g. food motivation (hunger, thirst), thermoregulatory needs, social/sexual interactions, escape from danger, etc. One could reason that important non-photic events, particularly when occurring at a regular interval, can be proficient to establish changes in circadian rhythmicity. A feedback mechanism of such cues could be of functional relevance adding to the light-driven entrainment. In the laboratory, stimulation of behavioural activity during the day, for instance by novel wheel exposure, is known to induce phase shifts in overt circadian rhythms. It is often difficult to elucidate the critical factor of such activity-induced phase shifts (see Mrosovsky et al., 1996), i.e. is the effect triggered by the activity itself (movement), by the underlying intrinsic motivation to become active, by arousal (sleep-to-wake transition), or by stress/anxiety?

Strikingly, the results described in **chapter 6** demonstrate that a mild intervention of the animal's rest, triggering behavioural activity, induces opposite responses as compared to spontaneous behavioural activity, i.e. acutely, but transiently, increased SCN electrical discharge. Although future research is required to unravel the critical underlying drive for this discrepancy, a stress effect is unlikely since cortisol levels increase rather slowly and stress has been reported to have minor effects on circadian system (Van Eekelen et al., 1987; Rosenfeld et al., 1988; Meerlo et al., 1997; Mistlberger et al., 2003). Alternatively, the phenomenon could be due to a 'wake-up' effect. The transition from sleep-to-wake was found to be associated with increased electrical activity levels in the rat (DeBoer et al., 2003), however, the kinetics were completely different as the neuronal activity levels continued at the elevated levels while the present data showed transient responses of maximal 200sec.

Photic and non-photic cues under natural conditions

The sensitivity to non-photic influences presumably differs between species. It has been reported that the rat is far less sensitive to non-photic stimuli than the hamster (Mistlberger et al., 1991). Sensitivity to photic or non-photic cues can change under different environmental conditions. For instance, under prolonged darkness the circadian system shows larger phase shifts (Refinetti et al., 2007), which may reflect increased sensitivity to light. Following two days of constant light, the system is greatly susceptible to non-photic cues and can respond with extraordinary phase shifts (Knoch et al., 2004). Also under changing photoperiodic conditions, the response to photic and/or non-photic cues may be different. This is of particular significance for animals that have their habitat in moderate to polar latitudes. The survival rates of these animals rely on the capacity to seasonally adjust their

physiology to the time of the year. Light exposure at dawn and dusk seems especially important for the winter-to-summer adaptation, when the light period extends day by day. Indeed, photic input is differentially effective in animals kept under short versus long photoperiod, with increased amplitude of the PRC to light under short day length as compared to long day length (VanderLeest et al., 2009; Pittendrigh et al., 1984). Altered sensitivity to non-photic input under short versus long photoperiod may be hypothesized as well, but limited evidence is available (Evans et al., 2004). When day lengths are gradually shortening as in the summer-to-winter scenario, the circadian system might benefit from enhanced sensitivity to non-photic cues rather than photic cues.

Photic and non-photic interactions

From most *in vivo* recordings from mouse SCN we could detect either light-induced responses or behavioural activity-induced responses of SCN electrical discharge; we rarely observed responsiveness of SCN firing to both stimuli. Histological determination of the location of the electrode tip revealed that the probability of measuring behavioural activity-induced suppressions was increased in placements in the dorsal SCN, while light responses were most likely observed in the ventral SCN.

The findings presented in this thesis illustrate that photic and non-photic cues are powerful stimuli for modulation of the SCN activity. Exposure to several photic or non-photic stimuli results in acute effects on the SCN electrical activity patterns. The circadian pacemaker is capable to detect several stimulus features, most importantly stimulus strength, duration, and timing, which is true for both photic and non-photic stimuli. Results from **chapter 4** and **chapter 6** show that light and behavioural activity have opposing effects on the SCN neuronal firing rate (see also Schaap and Meijer, 2001). Results from **chapter 5** demonstrate that light and opioids are mutually antagonistic and can block each other's phase shifts. Photic cues induce excitations, and non-photic cues elicit suppressions of the SCN electrical activity in nocturnal animals. In view of the endogenous circadian rhythm of the SCN, with high electrical activity levels during the day and low levels during the night, it can be reasoned that stimulus exposure at an 'unexpected' phase of the cycle (e.g. light during the night, and overt activity during the day) produces a shift of the circadian clock. Stimulus exposure at a 'common' phase of the cycle (e.g. light during the day and overt activity during the night), may have a reinforcing effect on the SCN rhythm amplitude. In conclusion, dependent on the phase of the cycle, the photic and non-photic cues serve to maintain, to enhance, or to fine-tune the phase and/or amplitude of the

rhythm. This reasoning implicates that a stable temporal relationship with the environment is regulated by the interplay between photic and non-photic stimuli.

Implications for health and circadian rhythm disorders

The primary focus of this thesis was to understand the mechanisms of photic and non-photic entrainment impulses which are at the base of circadian pacemaker modulation. Meanwhile, results from fundamental research may provide useful tools to improve health or well-being, and may help to develop strategies to encounter circadian rhythm disorders. Sleep and circadian rhythm disorders are prevalent in many physical and mental diseases, e.g. bipolar disorder, seasonal affective disorder, schizophrenia, metabolic syndrome, Alzheimer's disease, attention deficit hyperactivity disorder, Huntington's disease, diabetes mellitus, Parkinson's disease, chronic headache disorders, advanced/delayed sleep phase syndrome, Smith-Magenis syndrome, etc. Although the severity of circadian rhythm- or sleep-disorders are often correlated with the clinical stage of a certain disease, the causal link is not always clear. Whether or not the circadian rhythm disorder results from the pathology of the disease, or is involved in the etiology of the disease (Menet and Rosbash, 2011), treatments that focus on circadian functioning are often alleviating the clinical symptoms.

Biological clock dysfunction may involve internal desynchronisation of central and/or peripheral clocks, circadian rhythm amplitude reduction, and/or deficits within extra-SCN oscillators, amongst others. Several of these circadian topics have passed in this thesis, either directly or indirectly, and in this final paragraph the studies will be viewed in the context of clinical perspectives.

Light and exercise

Light has major beneficial implications for health, well being and cognitive functions. Moreover, light exposure is extensively reported to be effective in the treatment of circadian disturbances, for instance as observed in depression and Alzheimer's disease. **Chapter 4** discussed the differential influences of spectrum, irradiance and timing of light on circadian functioning. Knowledge on the relative impact of several light aspects can optimize the light treatment. In aged people, short wavelength light input is reduced due to yellowing of the lens/cornea. This may lead to improper circadian functioning and emphasizes the importance of a well-chosen spectrum of the light source.

Chapter 3 focused on the synchronization mechanisms underlying the physiological adaptation to new time zones. This relates to paradigms of rotational shift work and jet travelling across time zones, which typically lead to circadian rhythm disturbances involving internal desynchronisation. The pathways facilitating phase resetting to advanced time zones were shown to involve $Ca_V2.1$ channels, perhaps via specific neurotransmitters modulating G-protein coupled receptors. These signaling pathways may be useful clinical targets in the treatment of a misalignement between internal oscillators and the environment.

Appropriate timing of light exposure is strictly important to re-entrain, but the rate of re-entrainment can also be influenced by the extent and timing of physical exercise. Shifted rest-activity cycles of shift workers or of blind people may also affect the circadian rhythm amplitude, due to the acute suppressive effects of behavioural activity on the SCN pacemaker, as was discussed in **chapter 6**. A reduction in rhythm amplitude is the most prominent underlying cause of circadian rhythm disorders observed in the elderly and in Alzheimer patients. Damped amplitudes occur in rhythms in rest-activity, body temperature, hormone levels, and SCN firing rate (Van Someren et al., 1993; Nakamura et al., 2011). Chronobiological treatment aims to increase the rhythm amplitude. Both light and physical exercise can boost the rhythm amplitude when occurring at the right phase of the cycle (Van Someren et al., 1997a; 1997b; Van Someren, 2000; Teri et al., 2003; McCurry et al., 2005). The importance of reinforcement of the rhythm amplitude by appropriately timed physical exercise is highlighted in **chapter 6**.

Migraine and the circadian system

Chapter 3 links the circadian phenotype of a mouse model to the genotype of human familial hemiplegic migraine type I (FHM1). Migraine is associated with altered neuronal excitability within the brain. One of the consequences of the changed excitability is an increased susceptibility to cortical spreading depression (CSD), a propagating wave of neuronal depolarization, which is thought to be the underlying cause of migraine aura. In **chapter 3**, it was shown that hyper-excitability was also present in the synaptic contacts of afferent pathways within the SCN in migraine mice, confirming that the circadian system is directly affected by the mutation. The results described in **chapter 3** show that phase resetting to advanced time schedules is significantly influenced by the migraine mutation. In migraine patients, irregular rest-activity rhythms, jet lag, a lack of sleep, or prolonged sleep, can all trigger migraine attacks (Silberstein, 1992; Sahota and Dexter, 1990). Evidence accumulates that the circadian pacemaker is involved in the pathophysiology of migraine, and

perhaps other forms of chronic headache such as cluster headache (Zurak, 1997). Migraine, like cluster headache, exhibits circadian and circannual rhythmicity, with increased prevalence in the morning (Solomon, 1992; Pringsheim, 2002; Alstadhaug et al., 2008), and during summer time (Alstadhaug et al., 2007). Migraine patients have disturbed rhythms of melatonin, prolactin and cortisol (Ziegler et al., 1979; Ferrari et al., 1983). Of interest, the specific mutation of FHM has been reported in humans to be associated with a genetic deficit in the molecular clock machinery. As a result, these patients also suffered from advanced sleep phase syndrome, a circadian rhythm disorder that is characterized by entrained rhythms with extremely early sleep onsets and morning awakenings. Furthermore, migraineurs often have strong aversion to light, as light exacerbates the perception of migraine headache. This photophobia was recently suggested to be mediated by the melanopsin photopigment (Noseda et al., 2010). Finally, preliminary results from follow-up studies using EEG sleep recordings now have indicated that the R192Q migraine mice sleep significantly less as compared to control mice, at the expense of NREM sleep. These findings implicate that sleep regulation is also affected in R192Q migraine mice (Deboer et al., 2008).

Chronopharmacology

Since daily oscillations are present in numerous human bodily functions, ranging from cardiac functioning to blood flow and liver- and kidney functions, it is not surprising that these processes may influence various health aspects. Diurnal variations were also found for pain thresholds, with peak and trough times being dependent on type and cause of the pain (Junker and Wirz, 2009). Circadian rhythms in endogenous opioid activity and opioid receptor expression may underlie the perception of pain. Maximal analgesic effects of enodogenous endorphins usually occur during the activity period, both in mice and humans (Junker and Wirz, 2009). **Chapter 5** pays attention to the opioid fentanyl, which is a homologue of morphine and is a commonly used painkiller and sedative drug in clinical settings. Fentanyl was shown to directly modulate the SCN pacemaker activity. In humans, the analgesic effects of fentanyl were reported to follow a circadian rhythm, being maximal in the early morning, and minimal in the early evening (Boom et al., 2010).

Chronopharmacology studies have revealed circadian variability in the pharmacodynamics and -kinetics of several drugs (Bruguerolle, 1998; Levi and Schibler, 2007). For instance, pharmacokinetic parameters of various non-steroidal anti-inflammatory drugs vary as a function of dosing time (Clench et al., 1981; Bruguerolle, 1998), and significant daily fluctuations of serum concentrations have

been reported for several anti-asthma drugs and anti-cancer drugs, despite a constant intravenous infusion rate (Bruguerolle, 1998; Levi, 2010). Chronopharmacology is an important research area as the time of drug delivery can improve the outcome of pharmacotherapy, both by increased therapeutic efficiency and decreased drug toxicity.

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