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GENERAL INTRODUCTION

Many species in the plant and animal kingdom exhibit circadian rhythms in order to anticipate to daily changes in their environment. Circadian rhythms were first described in plants. In 1729 Jean-Jacques de Mairan described the presence of rhythms for the first time in the Mimosa plant. This plant followed the sunlight with its branches and leaves throughout the day. Most surprisingly at that time, these rhythmic movements continued in constant darkness. This finding indicates that external light is not necessary to maintain circadian rhythmicity.

Two hundred years later Maynard Johnson was able to record behavioral activity of mice and found a clear nocturnal activity pattern. Mice started activity right after sunset and ended just before sunrise. Similar to observations made by de Mairan in plants, mice kept their behavioral activity pattern of about 24 hours in constant darkness. Maynard made sure there were no environmental changes in the room apart from light. He was the first who concluded that mice have an intrinsic ability to regulate a rhythmicity of about 24 hours.

Since 1729, circadian rhythms have been determined in many species. However, the actual presence of a central pacemaker was not identified until 1972. In mammals the central pacemaker is located in the suprachiasmatic nucleus (SCN) of the hypothalamus. It was initially identified by specific lesioning of light recipient areas, including the suprachiasmatic area, right above the crossing of the optic nerves. Ablation of this area resulted in the complete disappearance of circadian rhythms in behavioral activity. The location of the central pacemaker was confirmed by transplantation studies in which restoration of circadian rhythmicity was observed after foetal SCN transplantion in SCN lesioned arrhythmic hamsters [1, 2]. The location of the pacemaker was also identified in species other than mammals. For example, in snails the clock appeared to be located in the retina [3], in fruitflies it is located in the optic lobes. In zebrafish no specific location of the pacemaker has been determined yet and the question is whether zebrafish do have a localized pacemaker or whether there is a more global organization in the circadian system of zebrafish.

THE SUPRACHIASMATIC NUCLEUS

The SCN in mammals is a bilateral structure containing approximately 10 000 neurons in each hemisphere. Individual SCN neurons spontaneously generate circadian rhythms in electrical impulse frequency [4]. Electrical activity levels of SCN neurons are high during the day and low during the night [5, 6]. 24-hour rhythms are generated at the molecular level in a transcriptional-translational feedback loop [7]. The molecular feedback loop leads to changes in membrane excitability, which results in rhythmic generation of action potentials. In isolation the SCN still expresses a rhythm in electrical activity indicating that the SCN does not need input from other brain areas to maintain rhythmicity [5]. Individual SCN neurons in culture

have widely distributed phases and they have their own period. It is essential for SCN neurons to be synchronized to be able to generate a coherent output signal. SCN neurons are synchronized to each other via communication through electrical coupling and neurotransmitter release. Several neurotransmitters are implicated in synchronization of SCN neurons, including gamma-aminobutyric acid (GABA) [8] and vasoactive intestinal peptide (VIP) [9]. VIP is expressed at high levels in neurons of the SCN [10, 11]. The loss of VIP results in reduced behavioral rhythmicity in constant conditions [12]. The application of VIP in cultured SCN cells induces phase shifts [13]. VIP is also important in transmission of light information within the SCN [14]. In addition, VIP plays a role in encoding photoperiodic information [15], a process that relies largely on changes in coupling within the SCN. For the communication within the SCN GABA was shown to be specifically important [16]. GABA is expressed throughout the SCN and exhibits both inhibitory and excitatory effects on neuronal activity. Synchrony of spiking across SCN neurons also occurs via electrical coupling [17]. Electrical coupling is regulated via communication through gap junctions. The incidence of electrical coupling between SCN neurons is about 26% [17-19].

SCN neurons form a heterogeneous population which can be divided in several regions and an important distinction does exist between the dorsal and the ventral region of the SCN. The ventral region is the most light-recipient part and receives direct light input from the optic nerve fibers [20]. Other afferents which terminate in the ventral part of the SCN are serotonergic projections from the raphe nuclei and neuropeptide Y (NPY)-releasing terminals from the intergeniculate leaflet (IGL). Ventral SCN cells mainly express VIP and gastrin releasing peptide (GRP). The dorsal region of the SCN mainly contains vasopressin (VP) and somatostatin expressing cells. Dorsal SCN neurons receive their major input from the ventral SCN, the hypothalamus and the forebrain. The GABAergic communication between the ventral and the dorsal region of the SCN is probably important for interregional synchronization within the SCN [21].

LIGHT TRANSDUCTION TO THE CLOCK

Light is the primary cue for the circadian system to detect daily changes in the environmental cycle. Light is sensed by rod- and cone photoreceptors in the outer retina and the photosensitive retinal ganglion cells (pRGCs) containing melanopsin in the inner retina. Each class of photoreceptors has its own maximum sensitivity to a specific wavelength of light. Murine cones have two sensitivity peaks; the short wavelength cone has its maximum sensitivity peak in the UV light range (λ_{max} 360nm)[22] and the mid wavelength cone is most sensitive to green light (λ_{max} 508 nm). Rod photoreceptors were also reported to have their highest sensitivity to green light (λ_{max} 498 nm)[23] and melanopsin is maximally

sensitive to blue light (λ_{max} 480nm)[24, 25]. Melanopsin causes pRGCs to be directly photosensitive. In addition to their intrinsic photosensitivity pRGCs receive indirect light input from rod- and cone photoreceptors [26, 27]. When the rod and cone input is blocked, pRGCs can still depolarize in response to light. However, the light response characteristics at the level of pRGCs show clear differences in the absence of rods and cones. The responses of pRGCs are remarkably slow and tonic, while the responses of rod- and cone photoreceptors are fast and transient [27, 28]. Both rod and cone photoreceptors and pRGCs can transmit photic signals to the SCN. Mice lacking melanopsin are able to entrain to light, demonstrating that rod- and cone photoreceptors are sufficient for circadian entrainment [29, 30]. However, mice lacking all rods and cones but still retaining melanopsin can yet entrain to light normally, showing that melanopsin can compensate for the loss of rods and cones [31]. Together these findings indicate that each class of photoreceptor can contribute to circadian photoentrainment.

Photic signals are transmitted through the retino hypothalamic tract (RHT) to the SCN. Axons of the retino hypothalamic tract orginate in the retinal ganglion cells and project monosynaptically to the ventral part of the SCN [32]. Light signals trigger the release of several neurotransmitters from RHT terminals, namely glutamate and pituitary adenylate cyclase-activating peptide (PACAP)[33]. The release of glutamate and PACAP excites SCN neurons and activates postsynaptic NMDA and/or AMPA receptors and PACAP type 1 receptors [34, 35]. Activation of these receptors leads to increased intracellular calcium levels which affect rhythms in clock gene expression [36].

In vivo extracellular recordings of SCN neurons show a tonic increase in electrical activity levels in response to retinal illumination [37-42]. A small proportion of neurons shows a decrease in impulse frequency after retinal exposure to light [39, 43-45] and are defined as light-suppressed neurons [46]. Other neurons show only a transient increase in response to light [45]. Light-activated neurons show typical response characteristics, with a transient increase in electrical activity when lights are switched on, followed by a tonic increase for as long as the light stimulus remains. When lights are switched off SCN neuronal activity is temporarily suppressed. The magnitude of the tonic phase of the light-induced increase in impulse frequency is dependent on the light intensity. [37, 39-42, 47]. Light-induced increases in SCN neuronal activity change over the time of the day. Light induces large increases in SCN neuronal activity during the night, which is the active phase of the mice, while only small responses are observed during the day [40, 48].

The increase in SCN neuronal activity in response to light is highly correlated with light-induced phase shifts in behavioral activity [49]. The effects of light on behavioral activity patterns can be best determined when animals are maintained in a constant dark environment and short pulses of light are presented at specific phases in the circadian cycle. Light exposure in the beginning of the active period

leads to a phase delay in the behavioral activity rhythm, whereas light exposure at the end of the active period induces a phase advance. When the animals are exposed to light during their resting period, no shift in behavioral activity occurs. This phase is referred to as the "dead zone". The time-dependent effects of light on phase-shifting of the circadian system are usually summarized in a phase response curve (Figure 1A). In addition to the time of the day, the magnitude of the phase shift in behavioral activity is dependent on the duration, wavelength and intensity of the light pulse [50]. The threshold for the duration of a light stimulus to induce a phase shift in behavioral activity is 30 seconds. The minimum irradiance to induce a significant phase shift is 100 billion photons (10^{11}) and the circadian system is maximally sensitive to 500 nm of light [51].

INFLUENCES OF LOCOMOTOR ACTIVITY ON THE CLOCK

While light is the main external cue for the circadian system to synchronize to its environment, the clock can also be entrained by non-photic factors such as behavioral activity, sleep and social stimuli. Behavioral activity can induce phase shifts of the circadian system. The magnitude and direction of the phase shift in response to behavioral activity is dependent on the time of the day (Figure 1B). This dependence is found by presenting a running wheel to an animal for a few hours, thereby enhancing behavioral activity. The effect of enhanced behavioral activity is evaluated in constant dark conditions. Large phase advances are observed following behavioral activity during the resting phase of the animal. Behavioral activity during the active phase induces small phase delays [52] [53]. Behavioral activity is reported to be an effective entrainment cue. Scheduled voluntary exercise can modify entrainment to a light-dark cycle, showing a large influence of behavioral activity on circadian activity patterns [54]. Reduced rhythmicity can be restored by making use of scheduled exercise in mice lacking VIP or its receptor [55, 56]. In addition, enhancing behavioral activity is effective in restoring rhythmicity in aged mice [57] and improves rest-activity rhythms in elderly [58].

Behavioral activity is thought to exert its effects on the SCN mainly via two afferent neuronal pathways. The geniculohypothalmic tract sends NPY-containing fibers from the intergeniculate leaflet (IGL) to the SCN. Lesioning of the IGL results in a reduction of phase shifts in response to behavioral activity [59], while electrical stimulation of the IGL enhances these phase shifts [60]. Administration of NPY also induces a phase shift in behavioral activity [61]. The second pathway is the serotonergic projection originating from the raphe nuclei. Serotonin is released in the SCN in response to behavioral activity [62]. Electrical stimulation of the raphe nuclei leads to phase shifts in behavioral activity [63] and serotonin release in the SCN [64, 65]. Administration of serotonin results in non-photic phase shifts [66]. Depletion of serotonin inhibits behavioral-induced phase shifts [67]. In the

SCN, serotonin affects neuronal activity by suppressing c-fos expression and SCN electrical activity [65, 68-70]. Extracellular *in vivo* recordings show acute effects of behavioral activity on SCN electrical impulse frequencies. Behavioral activity suppresses SCN neuronal activity levels [6, 71, 72]. The magnitude of the suppression in electrical activity is dependent on the intensity and duration of the behavioral activity [73].

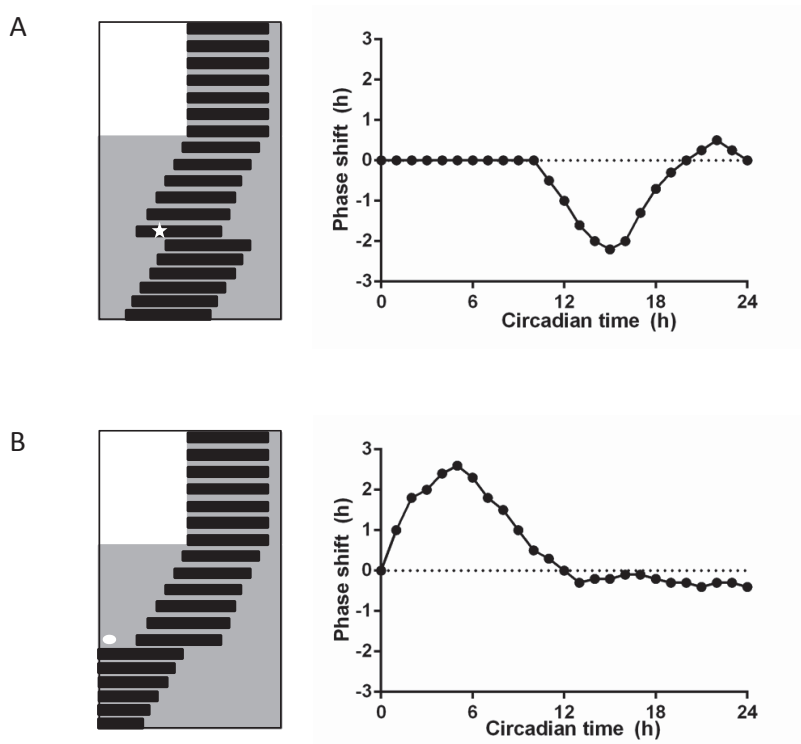

Figure 1. A. Schematic representation of a phase response curve (PRC) to light. On the left a schematic example of an actogram of mouse wheel running activity is shown. Consecutive days are plotted below each other. The black bars represent wheel running activity of a mouse. The white star on the sixth day in constant darkness represents light exposure at the beginning of the active phase, which causes a delay in the behavioral activity rhythm of the mouse on the following day. On the right the magnitude and the direction of the shift in behavioral activity are plotted as a function of circadian time. Light exposure in the beginning of the active phase of the mouse induces a phase delay, while light exposure towards the end of the active phase of the mouse results in a phase advance of the behavioral activity rhythm. B. Schematic representation of a PRC to enhanced behavioral activity by introduction of a running wheel. On the left an example of an actogram of a mouse is shown. The white circle represents the introduction of a running wheel, which induces a phase advance in the mouse behavioral activity rhythm on the next day. On the right the size and direction of the phase shifts are summarized in the PRC as a function of circadian time.

SLEEP AND THE CLOCK

Sleep is regulated by an interplay between the need for sleep and SCN functioning. After removal of the SCN, rhythms in sleep are disrupted, while the total amount of sleep in most animals remains the same [74, 75]. In addition, a correlation exists between electroencephalogram (EEG) recordings to measure sleep and SCN neuronal activity. SCN neuronal activity is affected by alternations between sleep states [76]. Extracellular neuronal activity recordings revealed that SCN neuronal activity decreases in response to an increase in sleep pressure [77](van Diepen, H.C., Meijer, J.H., Deboer, T., unpublished, Figure 2). Behavioral activity recordings showed reduced phase shifting capacity in response to light after sleep deprivation [78, 79]. One of the substances thought to be involved in sleep regulation is adenosine. Adenosine levels in the brain rise during sleep deprivation and decrease during recovery sleep [80]. Administration of adenosine agonists mimicked the effects of sleep deprivation on light-induced phase shifting [81-83]. These effects could be counteracted by administration of adenosine antagonists [82, 83]. Together, these findings clearly indicate a strong interaction between sleep regulation and the circadian system.

OUTLINE OF THIS THESIS

The research described in this thesis aims to understand light signaling from the retina to the SCN. The first part explores the relative contribution of the various photoreceptors in the retina to light responsiveness of the SCN. The second part focuses on the influence of VIP and adenosine on light sensitivity of SCN neurons. Finally the influence of exercise on the SCN will be evaluated.

Chapter 2 reviews light signaling from the retina to the SCN. Light is perceived by photoreceptors in the inner and outer retina, which activates a signaling cascade from the retina via the retino hypothalamic tract to the SCN. The circadian effects of light are discussed in this chapter at the level of behavioral activity as well as at the level of SCN neuronal activity. Chapter 3 focuses on the effects of UV light on the circadian system and sleep. Light is the main synchronizer of the circadian system and activates photosensitive cells in the retina. In contrast to humans, the retina of mice contains short wavelength sensitive cones, which are specifically sensitive to UV light. Particular attention has been paid to the effects of UV light on the circadian system in the absence of the photopigment melanopsin. Melanopsin was thought to be important for regulating the effects of light on the circadian system. In chapter 3 we investigate the effects of UV light on behavioral activity, sleep and SCN neuronal activity in the absence of melanopsin. The effects of long wavelengths of light on the circadian system are determined in chapter 4. In this chapter we focus on the ability of rod- and cone photoreceptors to mediate the

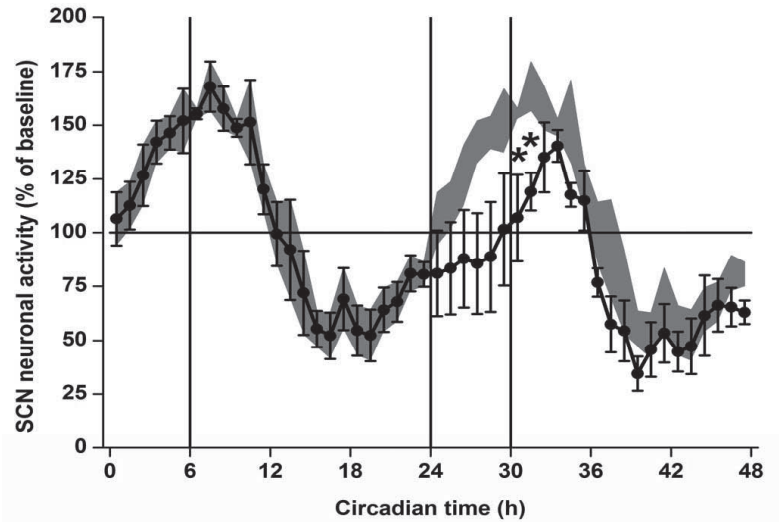

Figure 2. Mean *in vivo* electrical activity levels of SCN neurons of mice plotted as a percentage of baseline activity levels over 48 hours. SCN electrical activity levels are high during the day and low during the night. After a baseline recording of 24 hours, mice were sleep deprived between circadian time 24 and 30. The shaded area represents the baseline data ±SEM and the black line from circadian time 24 represents mean SCN electrical levels ±SEM after sleep deprivation. SCN electrical levels were significantly diminished after sleep deprivation. * indicates $p<0.05$, paired t-test after significant ANOVA.

effects of light on SCN neuronal activity. We explore these effects by making use of various retinal mutant mouse models, to extrapolate the relative contribution of rod- and cone photoreceptors to light regulation of the SCN. Our data indicates that cones predominantly mediate the acute light-induced increases in SCN neuronal activity, especially at high light intensities. In chapter 5 we test whether cone photoreceptors contribute to light responsiveness of the circadian system. This was done by investigating entrainment to various wavelengths of light in mice having cones as the only photoreceptors in their retina. In addition the acute effects of various wavelengths of light on SCN neuronal activity in these mice have been determined.

Factors that might influence light sensitivity of the SCN are investigated in chapter 6 and chapter 7. Chapter 6 explores the ability of caffeine to regulate light sensitivity of the SCN. Caffeine is an adenosine receptor antagonist and is worldwide the most used stimulant to reduce sleepiness. Adenosine levels rise during wakefulness and decrease during subsequent period of sleep. The effect of prolonged wakefulness on light responsiveness of the circadian pacemaker both at the level of behavioral activity and SCN neuronal activity are evaluated. To determine the possible involvement of adenosine on SCN functioning, we tested the effect of caffeine on

light responsiveness of the circadian system. Chapter 7 evaluates the role of the neurotransmitter VIP in light signaling within the SCN. Little is known about the role of VIP in light transduction to the SCN. VIP is necessary for phase shifts in behavioral activity in response to light, while it is not essential for the entrainment to a light-dark cycle. The mechanism underlying the deficit in light-induced phase shifting is investigated in a mouse model lacking VIP. The experiments described in this chapter focus on the acute effects of light on SCN neuronal activity, on calcium levels within the SCN and on clock gene expression in the absence of VIP.

While light is its main synchronizer, the circadian system can also be entrained by non-photic factors such as behavioral activity. In the chapter 8 of this thesis the effects of enhanced behavioral activity on the circadian system are determined. It was shown before that voluntary exercise can restore rhythmicity in circadian compromised mouse models. The question remains whether exercise can also exert beneficial effects on a strong oscillator. We asses this question by investigating the effect of voluntary exercise on the amplitude of the SCN in healthy young mice.

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